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# **Stabilization of peptide structure by non-covalent interactions**

Een wetenschappelijke proeve op het gebied van de  
Natuurwetenschappen, Wiskunde en Informatica

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# 1

## Introduction

*Amphiphilic peptides have recently gained much attention for the development of novel functional materials. The combination of hydrophobicity with hydrogen bonding makes these peptides into a versatile class of molecular building blocks with respect to the ability to influence assembly behavior and folding. Lipidated peptides, obtained through the introduction of one or more aliphatic chains to a peptide, are an important and readily accessible subset of amphiphilic peptides. In this chapter an overview of the current work on lipidated amphiphilic peptides, their self-assembly and other properties is presented.*

## **1.1 Introduction**

Structural proteins and protein-based materials are an important part of living systems. Secreted products such as silk and mussel ligation fibrils consist of well defined protein structures with high mechanical strength. Keratins are important for the build-up of hairs and nails and collagen fibers are the main component in animal connective tissues. Muscle tissue contains fibers of actin and myosin, the interaction between these fibers allows for muscle contraction. Actin and myosin fibrils are also an important component of the cytoskeleton, the cellular scaffold that not only provides cells strength and shape, but is also responsible for motility. This network of fibrils in the cell is essential for efficient transport processes and organization in the cell.

In some cases undesired protein structures are formed as a protective measure in the removal of misfolded proteins from the cell. In case of a misfolding event otherwise soluble proteins can aggregate into fibrillar materials.<sup>1,2</sup> Deposits of such material are believed to be involved in a number of disorders such as Alzheimer's disease, Creutzfeldt-Jakob and type II diabetes.<sup>3</sup>

The diversity and specificity found in protein based materials are possible due to the well-defined interactions between the protein building blocks of which these materials are constructed.<sup>4</sup> Of course similar non-covalent interactions, such as hydrogen bonding, hydrophobic and electrostatic interactions, are responsible for the folding of proteins in the first place. In case of assembly of protein building blocks specifically the interacting interface is of major importance; the actual contact area can be quite small compared to the whole surface of either protein. Furthermore, loops and other well-defined secondary structures are structural elements often present at these interfaces. Not only the amino acid sequence at the interface, but also the exact orientation of the amino-acids – and therefore the three-dimensional folding is important.<sup>5</sup>

In efforts to mimic and influence protein-protein interactions the specific amino acid side chain interactions, hydrophobic effects, and the specific spatial distribution have to be taken into account. An important field in the examination of protein interactions involves the synthesis of model systems. Besides small organic molecules also small peptide fragments have been used as model systems. In order to stabilize a specific fold in these peptides<sup>6</sup> and mimic discontinuous binding sites peptides are often used in combination with a scaffold.<sup>7-10</sup>

An important development is the use of peptide amphiphiles as a class of protein mimics and model systems. Especially, the assembly behavior of peptide amphiphiles has

attracted much attention.<sup>11</sup> Not only could the self-assembly behavior provide a starting point for peptide based structures, but the peptide amphiphiles could also be co-assembled with other components in order to introduce additional functionality and/or biocompatibility, leading to a bottom-up fabrication of new nano-materials.<sup>12</sup> Properties of assemblies can be easily controlled through choice of constituent amino acid residues.<sup>13</sup> However, still a lot of research will be required to fully grasp the mechanisms involved in *e.g.* folding and self-assembly of this type of molecules.

## 1.2 ***Classes of peptide amphiphiles***

Amphiphiles can be dissected into a water-soluble and a hydrophobic part. From a structural point of view different types of peptide amphiphiles can be distinguished.<sup>14</sup> A first category is that of amphiphiles consisting only of amino acids. Inspired by naturally occurring small peptidic amphiphiles, many artificial model peptides have been prepared. Especially amyloid fibril forming proteins have been the inspiration of many to design low molecular weight peptides that can produce amyloid-like assemblies.<sup>15,16</sup> These peptides are examined either as a model for a fundamental insight into the molecular mechanisms involved in this type of assembly or as a mere starting point in the construction of more complex nanoscale objects.<sup>17-25</sup> These amphiphilic peptides can be further divided into two types. The first class is comprised of peptides with a linear stretch of hydrophobic residues coupled to a polar head group that mostly is constituted by a number of polar amino acids. The second class consists of structures with alternating polar and apolar residues.<sup>26</sup>

A second category of peptide amphiphiles comprises so-called lipidated peptides; these are amphiphiles obtained through attachment of one or more alkyl chains on the side chains or at the C- and/or N-terminus, often inspired by nature's lipidated proteins. Lipidation of proteins plays an essential role in a variety of biological processes such as signal transduction, transport, fusion and structure.<sup>27-29</sup> The lipidation usually serves to anchor and localize proteins in order to create self-assembled structures that can perform their task.<sup>30</sup> Lipidation is a well known strategy to create membrane penetrating peptides as there are *e.g.* pore forming iturins,<sup>31</sup> toxins like magainins,<sup>32</sup> siderophores such as marinobactins and aquachelins,<sup>33,34</sup> lipopeptaibols, metabolites of fungal origin<sup>35</sup> and surfactins, bacterial cyclic lipopeptides well known for their exceptional surfactant power.<sup>36</sup> Synthetic peptides with one or more alkyl chains, often at the N- and/or the C-terminus, have been thoroughly investigated.<sup>37</sup> There is growing interest in their ability to organize and display

(bio-)functionality in assemblies<sup>38</sup> and on both artificial and natural bilayer membranes.<sup>15,39-42</sup> Furthermore, peptide and protein polymer hybrids show amphiphilic properties.<sup>43-45</sup>

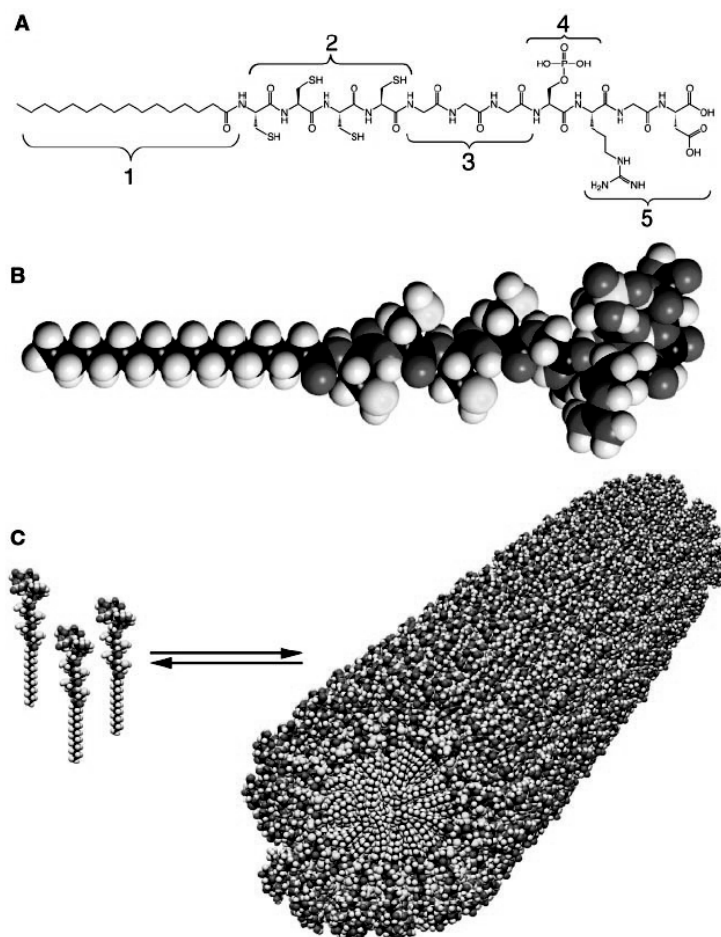
The remainder of this introduction will focus on lipopeptide amphiphiles, and more specifically on the assembly behavior of these amphiphilic peptides. A main division will therefore not be made on the molecular structure of the peptide amphiphiles, but on the type of assembly they are associated with: fibrous assemblies, monolayers and vesicular bilayers.

### **1.3 Peptide amphiphile fibrils**

Peptide amphiphiles based on lipidated peptides and amino acids were pioneered in the early eighties by the groups of Kunitake<sup>46,47</sup> and Yamada.<sup>48,49</sup> It was demonstrated that these amphiphiles are able to produce bilayer structures that can assemble into helical and tubular superstructures. The head groups of these amphiphiles contained either single amino acids or less defined oligomeric peptides obtained through a polymerization reaction. Later, Shimizu *et al.*<sup>50</sup> prepared well defined peptide amphiphiles with oligopeptides Sar<sub>3</sub> (N-Methylglycine) and Pro<sub>4</sub> as a head group. These compounds were shown to form bilayer based aggregates, presumably fibrous or ribbon-like structures that transformed into much smaller aggregates above their  $T_m$ . Elaborating on these structures Shimizu *et al.*<sup>51</sup> prepared a series of peptide amphiphiles each with a head group composed of different type and number of amino acids. Unsurprisingly, the phase transition parameters and aggregation morphologies were dependent on the nature of the head group, giving rise to not only tubular structures or twisted ribbons but also to vesicles and amorphous crystals.

More recently, the work of the group of Stupp gave a new boost to peptide amphiphiles in general and lipidated peptides in specific. Inspired by the way nature employs organic material as a scaffold for the control of the formation of inorganic materials,<sup>52</sup> Hartgerink *et al.*<sup>53</sup> demonstrated that a peptide acylated at the N-terminus with a single alkyl chain could be used in the construction of nano-scale materials suited as a scaffold for biomineralization. They described the reversible self-assembly of discrete micellar nanofibers via a pH controlled mechanism (figure 1). The fibers, after stabilization via disulfide cross-links, were able to direct mineralization of hydroxyapatite. The mineralized nanofibers were portrayed as comparable to the lowest level of hierarchical organization of bone and said to be easily modified through the selection of different amino acids for other applications in tissue engineering and mineralization. The pH controlled formation of peptide fibrils was used to assemble fibrils inside liposomal nano-containers.<sup>54</sup> After incorporation of the monomers in

the liposomes, a photo-induced pH change triggered fibril formation. Further *in situ* control on peptide fibrils was achieved through the introduction of a bulky photo-cleavable nitrobenzyl moiety. This group induced the formation of a quadruple helix superstructure that dissociated to single fibrils on exposure to light.<sup>55</sup>



**Figure 1.** A) General chemical structure of a peptide amphiphile used by Stupp and Hartgerink, highlighting five key structural features,. B) Molecular model of the peptide amphiphile, showing its conical shape. C) Schematic showing the self-assembly of the amphiphiles into a cylindrical micelle. Copyright 2001 AAAS.

Indeed, many variations on this type of amphiphile have been reported by the Stupp group, all self-assembling into nanofibers. A variety of subjects was covered using a combination of peptide amphiphiles,<sup>56,57</sup> mineralization of a metal sulphide semiconductor,<sup>58</sup> the encapsulation of carbon nanofibers,<sup>59</sup> ‘ink’ for dip-pen patterning<sup>60</sup> and catalysis.<sup>61,62</sup> They were even able to induce very rapid differentiation of cells into neurons, which was explained by the amplification of bioactive epitopes presented to the cells on the nanofibers.<sup>63</sup> Moreover, the peptide amphiphile nanofibers were suggested as possible vehicles in *e.g.* drug delivery. Probing the interior of the fibers revealed it was possible to capture small organic



molecules inside the core, which were still solvated enough to allow them to diffuse out of the assemblies again.<sup>64</sup> Furthermore, the nanofibers were also decorated with non-peptidic functionalities, such as biotin, allowing the binding to avidin.<sup>65</sup> Finally, azacrowns were introduced, enabling the binding of Gd(III) ions to be used as MRI contrast agents;<sup>66</sup> fluorescent labels were introduced for transfer studies.<sup>67</sup>

The use of self-assembled peptide amphiphile structures as scaffolds for the construction of inorganic nanotubes was studied by the group of Shimizu.<sup>68</sup> Nanotubular peptide self-assemblies were used as a template to construct CdS nanotubes.<sup>69</sup> Furthermore, it was shown that the binding of di- and tri-valent metal ions to Gly-Gly and Gly-Gly-Gly based peptide amphiphiles could be used to easily produce metal-functionalized nanotubes, which were converted into completely inorganic nanotubes by calcination.<sup>70</sup>

Lee *et al.*<sup>71</sup> studied peptide amphiphiles to explore their use in controlled release drug delivery. They prepared tripeptides coupled to glutamic acid dialkyl amide with alkyl chains of varying lengths. The helical ribbons and tubular structures they observed were coined high axial ratio microstructures (HARM). Incubation with liposomes or fetal calf serum suggested that the assemblies would survive long enough to be useful as drug delivery vehicles. A similar peptide amphiphile containing a decapeptide, spontaneously assembled into hollow tubes and helices.<sup>72</sup> Interestingly, the peptide was found to be significantly protected against degradation by trypsin, which was thought to be due to the tight molecular packing and folding of the peptide in the bilayer aggregates compared to that of dispersed peptide amphiphiles. The protection against proteolysis was considered to be a useful feature providing the assemblies with a potentially prolonged lifetime *in vivo*.

The conjugation of a lipid tail to a peptide not only has an impact on the folding and morphology of the formed aggregates, but also on the way in which the peptides prefer to assemble.<sup>73</sup> The A $\beta$ (16-22) peptide, KLVFFAE, assembles into fibrils and nanotubes.<sup>74,75</sup> However, Gordon *et al.* demonstrated with the use of solid-state NMR techniques that while the unmodified peptide formed anti-parallel  $\beta$ -sheets the same peptide with merely an N-terminal octanoyl moiety formed parallel sheets.<sup>76</sup> The authors suggest that this demonstrates that the amphiphilicity is critical in determining the mode of assembly and structural organization of the  $\beta$ -sheets that form and may provide a method for controlling the structure of amyloid fibrils.

Over the last decade Tirrell and Fields<sup>77-85</sup> have shown how to exploit the dynamic structure of bilayers to have peptide amphiphiles self-assemble into biologically active

protein-like structures. They showed that already a single N-acylation of a peptide with a hydrophobic moiety can be used to induce protein-like structures. *E.g.* a peptide, derived from a model peptide for  $\alpha$ -helices, did not form any distinct structure in solution. However, once connected to just a C<sub>6</sub> alkyl chain an  $\alpha$ -helical structure was adopted. Expectedly, the longer the alkyl chain of the modified peptide the higher the thermal stability of the assemblies that formed compared with the C<sub>6</sub> analogue. The increase of the temperature of  $\alpha$ -helix to coil transition could be correlated to the extent of aggregation. A similar stabilization was also shown with a 39-residue peptide, derived from type IV collagen which is prone to form triple-helix assemblies. Again, N-acylation with hexanoic acid already improved the stability of the triple helices. Furthermore, it was found that simple alkylation of peptides like (Gly)<sub>n</sub> (n=1-5) with a C<sub>18</sub> alkyl chains resulted in the formation of helical structures, which the peptides would not form otherwise. The stabilizing effect on peptide secondary structure by the attachment of hydrophobic alkyl chains to peptides seems to be a concept that might be utilized for studying the mechanisms of peptide folding and for the formation of more complex protein-like structures and peptide-based assemblies.

Similarly, a secondary structure was induced in an otherwise unordered, biologically active sequence derived from a matricellular protein, *i.e.* SPARC(119-122).<sup>86</sup> The activity was found to be enhanced while the susceptibility towards proteolysis was reduced. The results indicated amphiphilic surfaces can be designed to combine desired activities and select out undesired ones. Along this line an application of simply palmitoylated peptides as antifungal and antibacterial agents has recently been reported by Avrahami *et al.*<sup>87</sup>

In recent years van Hest and Löwik have shown that the introduction of an alkyl tail to the non-self-assembling Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly peptide causes the resulting peptide amphiphile to self-assemble in water; moreover, the morphology of the assemblies depends on the length of the hydrophobic tail.<sup>88</sup> With tails of C12 and shorter micellar assemblies prevail, while longer tails induce the formation of fibrils. These fibrils can be disassembled by heating the solution. However, these assemblies can be stabilized by the introduction of a di-yne containing alkyl tail and subsequent photopolymerization.<sup>89</sup> This photo-polymerization of di-yne moieties organized by peptide amphiphile self-assembly was subsequently also shown by Stupp *et al.*<sup>90</sup>

## 1.4 Monolayer assemblies

Biological membranes are important for the organization of functionality.<sup>91</sup> Monolayers at the air-water interface can be regarded as simplified two-dimensional systems useful as models for fundamental understanding on *e.g.* the relation between the morphology of monolayers and the chirality of its constituent components<sup>92</sup> but also for the construction of functional surfaces exploited in a variety of devices. The introduction of a peptide into a monolayer can be readily achieved by lipidation.

The most widely employed means of lipidation is the attachment of one or more alkyl chains at either the N- or C-terminus, as pioneered by Ringsdorf<sup>93</sup> and Kunitake.<sup>94,95</sup> The first group used the N-terminal part of a lipoprotein of *E. coli*, tripalmitoyl-S-glycerol-L-Cys-Ser, and studied its behavior and that of longer analogous peptides at the air-water interface in order to obtain information about *e.g.* their specific surface area and miscibility. The compounds were also proposed to be useful as low-molecular weight carrier-adjuvant systems evidenced by their mitogenic response towards B-lymphocytes.

The ability to recognize water soluble peptides at the air-interface was demonstrated by Cha *et al.*<sup>96-98</sup> They claimed that peptide amphiphiles can form ordered arrays of functional groups at the interface enabling binding events by the display of self-assembled complementary binding sites. Typically, it was found that single alkyl chain oligoglycines were not able to bind peptides, which was ascribed to strong inter-peptide hydrogen bonds preventing the intercalation of other peptides and thus hampering their binding.<sup>99</sup> Such intermolecular forces have been quantified on related compounds by Schneider *et al.*<sup>100,101</sup> The binding strength was increased by mixing in guanidinium based amphiphiles which are able to strongly interact with the carboxylate terminus of the ligands and to form antiparallel  $\beta$ -sheet like hydrogen bonds.<sup>102</sup>

The concept of molecular recognition by self-assembled binding sites at the air-water interface was further elaborated and examined in a combinatorial set-up by Huo *et al.*<sup>103</sup> Peptide libraries were constructed and spread on the air-water interface and the surface behavior was studied varying the solutes contained in the subphase. The authors claimed that the peptide lipids self-assembled at the air-water interface to form protein-like supramolecular structures capable of selectively recognizing specific ligands. Later, the ability of similar peptide lipids to form complexes with metal ions like  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  was investigated.<sup>104</sup> The binding performance was affected by the nature of the cations. Hard metal ions, like  $\text{K}^+$  and  $\text{Mg}^{2+}$ , clearly exhibited different binding activity than borderline and

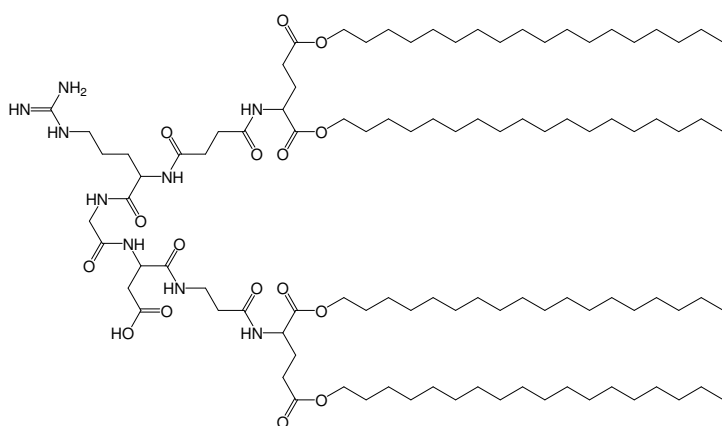
soft ions. Furthermore, based on fluorescent probe studies,  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  in the subphase were found to result in different behavior of the monolayers, supposedly due to a different mode of binding of the ions, leading to altered folding and thus assembly of the peptides in the monolayer. Both studies might well prove to be a starting point of a novel approach in the design of receptor molecules, termed combinatorial surface chemistry, to yield unprecedented protein-like supramolecular structures.

With the aim to construct responsive monolayers Huo *et al.*<sup>105</sup> incorporated a diacetylene functionality in the hydrophobic alkyl tail. It was found that the chromatic properties of the polymers formed after polymerization of the diacetylene moieties were indicative of the packing of the peptide amphiphiles at the interface. The area per molecule was nicely correlated with the maximum of the absorption peak in UV-VIS, shifting from 640 nm for a Gly-Gly head group to 525 nm for a Phe-Gly head group. In this respect the incorporation of a tryptophan residue was suggested to be useful as fluorescent reporter unit.<sup>106</sup> The formation of strong inter-peptide hydrogen bonds between the peptide head groups, as found by Cha *et al.*,<sup>99</sup> was further examined in the group of Leblanc using a pentapeptide based on a fragment of the  $\beta$ -amyloid peptide.<sup>107-111</sup> The aggregation process was monitored by surface pressure and surface dipole moment measurements combined with epifluorescence. It was shown the peptide was able to aggregate during compression and that the obtained topographies of the aggregates were explained by the peptide moieties.

An approach to design receptors based on protein-like supramolecular structures was thoroughly investigated by Tirrell and Fields.<sup>77-80,82,84,85,112,113</sup> Tirrell e.g. studied monolayers containing a peptide amphiphile based on RGD the so-called universal recognition site present in a variety of proteins that interact with cell-surface receptors of the integrin family. The amphiphilic peptide was mixed with a PEG-lipid and the structure of the resulting monolayers was studied employing neutron reflection. The head group was found to be perpendicularly oriented to the air-water interface; incorporation of the amphiphile into a PEG-lipid monolayer enhanced the configurational constraints.<sup>113</sup> The introduction of a short PEG spacer between an RGD tripeptide and the anchoring aliphatic moiety increased the proliferation of mouse fibroblasts on a monolayer while an aliphatic  $\text{C}_2$  spacer did not have this effect.<sup>114</sup> This shows that the whole amphiphile is important in the recognition process, not only the functional peptide.

In an attempt to gain further control over peptide folding Pakalns *et al.*<sup>115</sup> attached doubly alkylated glutamic acid derivatives to both the N- and C-terminus of an RGD

tripeptide to afford a compound which can be anchored with both termini, restricting its conformational freedom (figure 2). Most approaches to display peptides immobilize the peptide by covalently linking it through the N-terminus, leaving the carboxy-terminus free. It is a well-known fact, however, that many peptides exist in a conformationally constrained loop. It has been demonstrated that cyclic peptides which contain the RGD sequence can display higher affinities than their unconstrained linear counterparts. These peptide amphiphiles were utilized to prepare self-assembled monolayers which could be deposited as Langmuir-Blodgett films on a surface. On these surfaces functionalized with looped RGD amphiphiles melanoma cell were able to spread in a concentration dependent manner. Jensen *et al.*<sup>116</sup> showed a high biological activity at low incorporation levels using a cyclized RGD peptide with only N-terminal alkylation. Based on these results, they stated that engineered lipopeptide-based surfaces offer unique presentation opportunities that cannot be achieved by any other means of immobilization. Ochsenhirt *et al.*<sup>117</sup> not only showed synergy between amphiphiles based on RGD and PHSRN, but also showed a selective binding of linear and cyclized RGD sequences to different integrins.



**Figure 2 .** An N- and C-terminally lipidated 'looped' RGD peptide.

In an interesting approach Kros *et al* modified the water soluble amphiphatic (Leu-Glu)<sub>x</sub> peptide with a phospholipid moiety. The resulting amphiphilic peptide formed a stable monolayer on the air-water interphase for 3 and 4 repeats of the Leu-Glu sequence.<sup>118</sup> In this monolayer the peptide was organized in an antiparallel  $\beta$ -sheet. Furthermore, it was shown that these monolayers could be used as template for the mineralization of calcium carbonate.<sup>119</sup>

The use of peptide amphiphiles in combination with liquid crystalline materials in sensor applications was examined by Park *et al*.<sup>120</sup> Peptide amphiphiles were created by coupling a 17 amino acid peptide to amphiphiles that were interacting with a 4-cyano-4'-pentylbiphenyl (5CB) liquid crystalline (LQ) domain. This immobilization resulted in a stabilized loop conformation of the peptide. Furthermore, the introduction of the peptide resulted in a change in the LQ domain from planar to homeotropic alignment. The change in LQ phase could be reversed by cleaving the loop of the peptide with trypsin.

## 1.5 Vesicles

Peptide amphiphiles usually do not self-assemble into vesicular structures, they can however be readily integrated into for example liposomes. Integrins seem to be a suitable target for peptide functionalised liposomes because they serve as a model in many studies of cell-cell and cell-substrate interactions.<sup>121</sup> Recently, Waterhouse *et al*.<sup>122</sup> prepared a peptide sequence (PLAEIDGIELA) that targets  $\alpha 9\beta 1$ -integrin. Interestingly, they used cholesterol as a hydrophobic moiety, instead of an alkyl chain, and showed its applicability in gene delivery. However, most research has focused on introducing the arginine-glycine-aspartic acid (RGD) sequence.

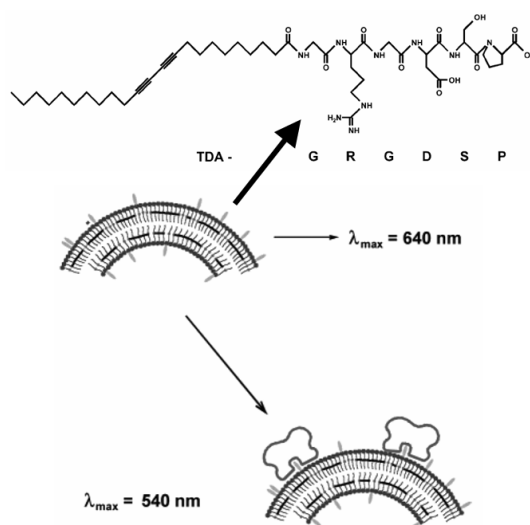
Hojo *et al*.<sup>123</sup> reported the synthesis of a glycerol derivative containing the cell-adhesion sequence RGDSG, as an archaeobacterial lipid model. They showed the conjugate was able to form stable liposomes. Yagi *et al*.<sup>124,125</sup> prepared liposomes whose surface was modified with peptides containing a five-time repeat of the GRGDS sequence, as found in the cell adhesion sequence of fibronectin. The peptide was lipidated by incorporation at the N-terminus of an aspartic acid residue modified with two C<sub>16</sub> alkyl chains. The availability of the peptides on the surface was confirmed using immuno-electron microscopy, employing a specific antibody to the peptide that could be visualized with gold colloids. The liposomes were shown to bind mouse fibroblast cells. The association takes place through interaction of the exposed peptide and the corresponding cell surface receptor.

Many different sequences have been anchored to liposomes to promote targeting to specific cells. In many cases a PEG chain is inserted between the peptide and the hydrophobic anchor, but at times more simple peptide amphiphiles are used.<sup>126,127</sup> For example, collagen based peptide amphiphiles have been introduced to induce a selectivity towards melanoma.<sup>128</sup> In addition to these specific targeting sequences, aspecific cell penetrating peptides have been used to stimulate uptake of liposomes into the cell.<sup>129,130</sup>

The group of Waldmann has extensively examined the role of hydrophobic alkyl chains attached to proteins.<sup>27,131</sup> Moreover, they have developed practical routes to synthesize peptides lipidated via thioesters, derived from a number of lipoproteins such as eNOS (NO-synthase),<sup>132</sup> Ras (guanine nucleotide binding protein) or hemagglutinin (glycoprotein from influenza virus A).<sup>133,134</sup> In the latter case, *e.g.*, they prepared peptides with S-palmitoylated cysteines. They showed that one single alkyl chain cannot prevent a peptide from hopping from one membrane to another, where two will. A recent alternative approach to prepare S-palmitoylated peptides, without the use of enzymes, has been described by Rijkers *et al.*<sup>135</sup>

To increase the activity of a peptide ligand often the structure is constrained fixing it in an active conformation. This strategy was employed by Marchi-Artzner *et al.*<sup>136</sup> who attached a cyclized RGD containing pentapeptide to lipid alkyl chains connected through a short ethylene glycol spacer (figure 12). Giant vesicles functionalized with these cyclic RGD peptides adhered to endothelial cells of the human umbilical cord, a process that could be inhibited by adding the corresponding soluble peptide. This suggests a specific interaction between the bilayer anchored peptide and the integrin receptors of the cells.

The RGD sequence has also been employed to construct vesicles that can be used to detect and report the interaction with an integrin. This can be accomplished by making use of the chromism of polydiacetylenes, which can be incorporated in vesicular bilayers.<sup>137</sup> Biesalski *et al.*<sup>138</sup> adopted this method, developed by Charych *et al.*,<sup>139</sup> for vesicles assembled from an RGD containing peptide amphiphile with a diacetylene lipid tail. It was shown that activated  $\alpha 5 \beta 1$  integrins in solution produced a chromatic shift in the polymerized vesicles, visible as a color change of the solution (figure 3).



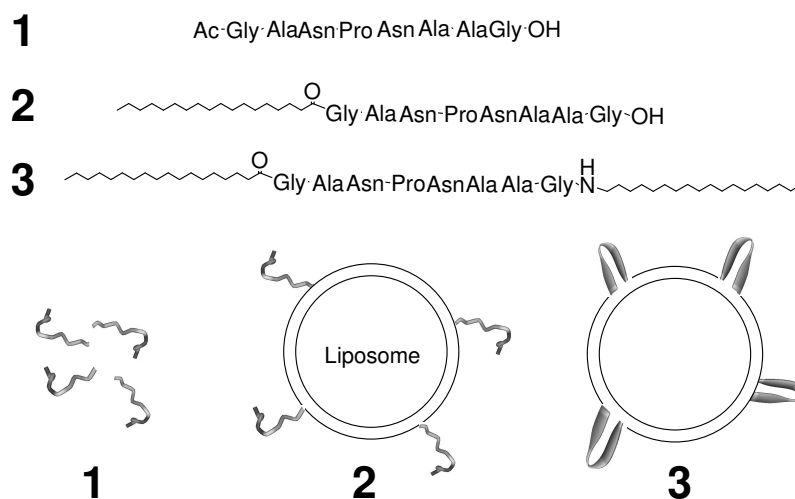
**Figure 3.** Structure of an RGD-diacetylenic peptide amphiphile and a schematic description of the molecular recognition of the peptide ligands located on the surface of the polymerized vesicles by the  $\beta 5\alpha 1$  integrin in solution. Copyright 2005 American Chemical Society.<sup>138</sup>

Another constrained peptide that was used as a head group in a peptide amphiphile is a cyclic NPNA derivative, a tetrapeptide that is found in a repeat region of the Circumsporozoite (CS) protein of the Malaria parasite *Plasmodium falciparum* in which it adopts a  $\beta$ -turn. Robinson and Pluschke<sup>140,141</sup> displayed this epitope on the surface of so-called immunopotentiating reconstituted influenza virosomes (IRIV) as an approach towards new designed synthetic vaccines. The conformational constraint was imposed upon the peptide by the incorporation of an *L*-Pro-*D*-Pro or an analogous peptidomimetic template that induced a hairpin. The approach was even extended to the preparation of a 35-membered library of cyclic peptide amphiphiles displaying a part of the AMA-I (apical membrane antigen-I) found on the surface of *Plasmodium falciparum* merozoites.<sup>142</sup>

Löwik *et al.*<sup>143</sup> demonstrated that the dynamic bilayer of a liposome can also be exploited to limit the conformational freedom of a peptide in non-covalent manner. They showed that the attachment of C<sub>18</sub>-alkyl chains to both the N- and C-terminus of a peptide allowed anchoring of both termini of the peptide into a liposome and thus forcing the peptide to adopt a  $\beta$ -hairpin fold (figure 4), similar to the work reported by Pakalns *et al.*<sup>115</sup> on monolayers of RGD containing looped amphiphiles described above. The peptide was easily prepared via an entirely solid phase route, starting with a commercially available aldehyde resin that was reductively aminated with the appropriate alkylamine. Similar to the work of Robinson and Pluschke<sup>141</sup> described above, a peptide was used containing a similar NPNA



sequence. It was shown that both the unmodified peptide in solution and the peptide containing only one alkyl chain, inserted into liposomes, showed random coil behavior, different from the doubly functionalized peptide that displayed  $\beta$ -hairpin characteristics once anchored in a lipid membrane. It was proposed that this simple approach can be conveniently employed for stabilizing a variety of peptides into a  $\beta$ -hairpin fold and might be used in the presentation of multiple properly folded epitopes.



**Figure 4.** Stabilization of a  $\beta$ -hairpin fold through the attachment of an N- and C-terminal anchor. Copyright 2003 RSC.<sup>143</sup>

The necessity of stabilization of peptide structures, and the possibility of achieving this through non-covalent interactions was nicely shown by Muhs *et al.*<sup>144</sup> They investigated two liposome based vaccines against the A $\beta$  amyloid sequence. Hydrophobic tails were introduced at both N- and C-terminus of the A $\beta$  1-15 fragment and the fragment was incorporated in a liposome. Liposomes were also functionalized with the A $\beta$  1-16 fragment through PEG anchors at both termini. The directly palmitoylated peptide was effective, in contrast to the PEG-anchored variety. The authors attributed this difference to a difference in the conformation of the peptide depending on the anchor used. The directly anchored molecule was present in a  $\beta$ -sheet conformation, while the PEG-functionalized peptide was present in a random coil conformation.

## 1.6 Thesis

The goal envisaged at the start of the research described in this thesis was the synthesis of protein mimics by spontaneous non-covalent assembly of peptides. The assembly process should not only be able to bring the building blocks together, but also stabilize the peptides in a biologically relevant conformation. As shown in this introduction one process capable of answering these demands is the (self-) assembly of peptide amphiphiles. Tuning of this self-assembly process should be possible by tuning the hydrophobicity of peptide amphiphiles. This thesis describes the influence of added hydrophobicity, achieved by the introduction of alkyl tails of varying lengths, on peptide (self-) assembly and folding.

In chapter 2, the influence of additional hydrophobic interactions on a peptide that forms amyloid like fibrils, with the sequence Lys-Thr-Val-Ile-Ile-Glu, is described. Specifically the effect of the introduction of an alkyl tail at the N-terminus or C-terminus on the stability of fibrils formed by the peptide is investigated.

In chapter 3 it is examined whether it is also possible to not only stabilize, but also disassemble fiber assemblies by manipulating the hydrophobic – hydrophilic balance. Furthermore, the possibility of *in situ* disassembly of peptide fibrils through the introduction of a cleavable hydrophobic moiety is studied.

In chapter 4 the influence of introduced hydrophobic interactions on the folding and assembly of a non-self assembling peptide, with the sequence Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly (GANPNAAG), is described. This non-assembling peptide has the propensity to fold in a  $\beta$ -turn.

In chapter 5 a series of alkyl modified GANPNAAG peptides is synthesized and anchored into liposomes. The effect on folding and anchoring is examined, by varying the length of the alkyl tails on the peptide and liposome composition..

Finally, in chapter 6, the possibilities are investigated to use alkyl modified GANPNAAG peptides, either anchored in liposomes or attached to a carrier protein, for the production of vaccines.

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# 2

## Stabilization of peptide fibrils by hydrophobic interaction<sup>‡</sup>

*Hydrophobic interactions play an important role in assembly processes in aqueous environments. In the case of peptide amphiphiles hydrophobicity is combined with hydrogen bonding to yield well-defined peptide-based aggregates. In this chapter we report a systematic study after the role of hydrophobic interactions on both stabilization and morphology of a peptide fibrillar assembly. For this purpose alkyl tails were connected to a known  $\beta$ -sheet forming peptide with the sequence Lys-Thr-Val-Ile-Ile-Glu (KTVIIE). The introduction of n-alkyl groups lowered the critical assembly concentration and induced thermal stability to the assemblies, without affecting the morphology of the peptide aggregates.*

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<sup>‡</sup> Part of this chapter has previously been published: J.T. Meijer, M. Roeters, V. Viola, D.W.P.M. Löwik, G. Vriend, J.C.M. van Hest, *Langmuir* **2007**, 23, 2058.



## 2.1 Introduction

The assembly behavior of peptides can be strongly influenced by the presence of amphiphilicity.<sup>1</sup> In principle there are two methods for the introduction of amphiphilic character into peptides. Firstly a peptide can be built up out of a combination of hydrophobic and hydrophilic amino acids, either in alternating or block sequence.<sup>2</sup> A second well-studied method is based on the attachment of alkyl-tails,<sup>3-5</sup> which allows even peptides that normally do not self-assemble to yield stable aggregates.<sup>6,7</sup> A single glutamic acid can e.g. form fibrils when coupled to stearic acid.<sup>8</sup> Not only self-assembly can be induced via the attachment of alkyl-tails,<sup>9-11</sup> but it can also change (the orientation of) the secondary structure<sup>12,13</sup> stabilizing a peptide in its bioactive conformation.

Peptide amphiphile based fibrils that are formed via this spontaneous assembly process have proven to be very useful, for example as scaffolds for cell growth or as template for the build-up of materials.<sup>5,6,14</sup> Self assembly of amphiphilic peptides and proteins is also important in biological processes; exposure of hydrophobic patches in proteins has been linked to amyloid formation.<sup>15</sup> The increase in solvent-exposed hydrophobic regions precedes insolubilization and aggregation of the protein. This spontaneous transition of soluble proteins into amyloid fibrils is a major cause of several disorders such as Alzheimer's disease<sup>16,17</sup> and diabetes type II.<sup>18</sup> Investigating and influencing this aggregation process, which is based on a combination of hydrophobic interactions and  $\beta$ -sheet forming peptide sequences, can contribute to the understanding of fibril formation and can possibly be useful for drug development.<sup>19</sup> Because of this biological relevance, much research has been devoted in recent years to study model peptides that self assemble into  $\beta$ -sheet like structures.<sup>20-22</sup>

The ability to induce stability to soluble peptide aggregates via the introduction of alkyl chains has remarkably so far only been applied to coiled-coil peptides<sup>13,23</sup> and non-aggregating peptides.<sup>24,25</sup> In the research presented in this chapter our main focus is on the effect of alkyl modification on peptide assembly of a known  $\beta$ -sheet forming peptide. We would like to know whether hydrophobic interactions could influence the stability of the peptide aggregate without affecting the existing peptide conformation. As a model peptide, the hexapeptide KTVIIE (K = Lysine, T = threonine, V = valine, I = isoleucine, E = glutamic acid), as designed by Serrano *et al*<sup>21</sup> was chosen, which is known to mimic amyloid fibril forming proteins. This model peptide is one of the smallest peptide sequences to form stable  $\beta$ -amyloid fibers at a wide pH range.<sup>26</sup> The peptide is small and can be easily and quickly synthesized. Furthermore, as previously published, the peptide sequence and charges in the

side chains are important for the self-assembly into fibrils.<sup>21</sup> The N-terminus of the model peptide is acetylated and the C-terminus amidated to limit the amount of charged states. Therefore, other functionalities can be introduced without disturbing either the peptide sequence or the charge that is responsible for the assembly process. This renders this model peptide an ideal candidate to systematically vary both the length and the position of alkyl chains, and study in detail the effect on assembly with respect to concentration and temperature dependence.

## **2.2 Synthesis**

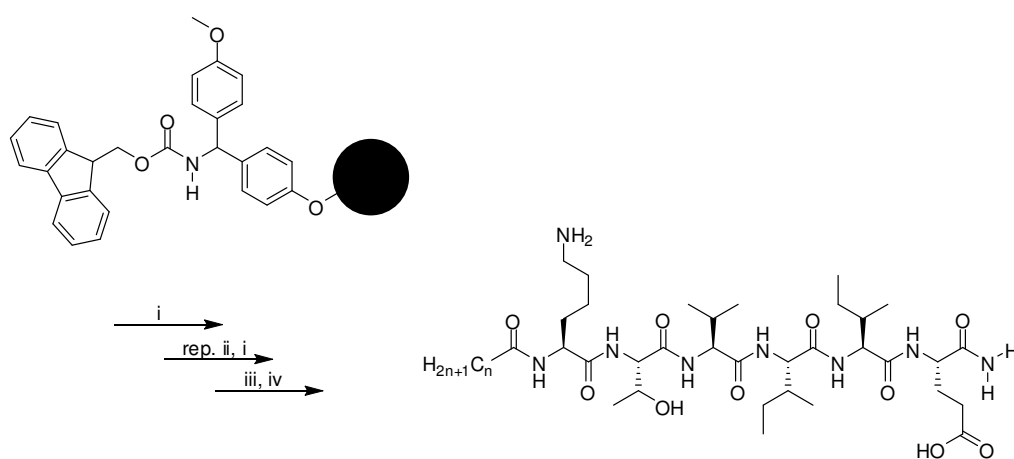
### **2.2.1 Synthesis of peptide amphiphiles**

Peptide amphiphiles were constructed with an aliphatic alkyl chain at either the N- or C- terminus of hexapeptide KTVIIE. For the N-terminal modified peptides the synthesis commenced on a solid support using a Breipohl amide resin (Figure 1).<sup>27,28</sup> Fmoc protected amino acids were coupled using the carbodiimide DIPCDI coupling agent with HOBt in DMF. Subsequently the Fmoc group was repeatedly removed using a 20% solution of piperidine in DMF. After the final amino acid was coupled, the alkyl chain could be introduced efficiently by a classical carbodiimide mediated coupling with the corresponding aliphatic carboxylic acid. This final coupling was performed in a dichloromethane/DMF mixture; the aliphatic tail was dissolved in dichloromethane, and neat DIPCDI and HOBt dissolved in DMF were added. Especially the longer aliphatic tails are more soluble in dichloromethane, and coupling efficiencies were found to be higher when this cosolvent was applied. Still, yields of the peptides with longer tails were lower, probably due to losses as a result of the higher hydrophobicity and thus solubility during the work up procedure involving precipitation in diethyl ether. (Table I, **1, 2, 5-9**).

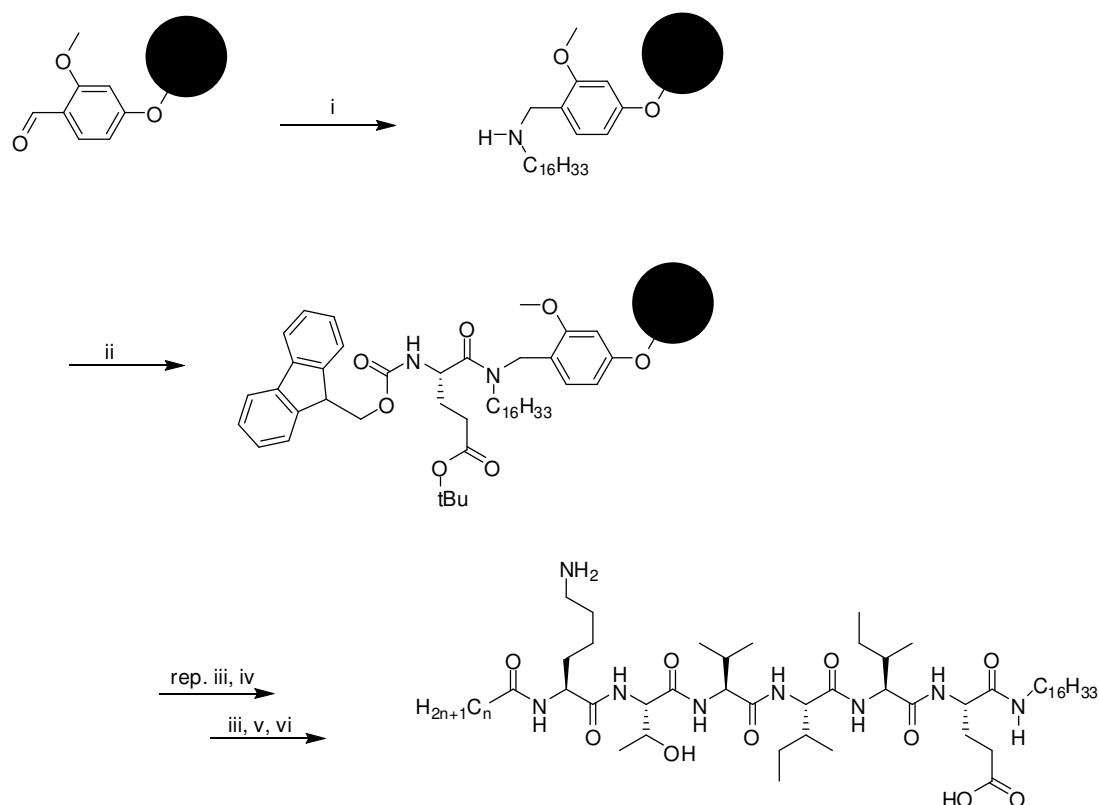
Alternatively, an aliphatic moiety was introduced on the C-terminus of the peptide (Figure 2). For this purpose an aldehyde functional resin was used and a coupling procedure was followed,<sup>29</sup> which was adapted from a method developed by Barany *et al.*<sup>30,31</sup> A primary aliphatic amine was coupled to the aldehyde resin by means of a reductive amination and the resulting secondary amine was subsequently the starting point for peptide synthesis. This versatile method can be used to introduce a wide variety of functional groups at the C-terminus of a peptide, such as fluorescent groups, aliphatic tails, crown ethers and even

polymers.<sup>5,29,32</sup> Introduction of the first amino acid to this secondary amine was slow. To ensure complete coupling an extended coupling procedure was used. The first amide coupling was performed using DIPCDI and HOBt in DMF. After an hour the solution was removed, and replaced with a fresh batch of reagents, after which the reaction was continued for another 15 hours. A chloranil test<sup>33</sup> was performed to ascertain the completion of the reaction. Any possibly remaining free secondary amines were capped by treatment of the resin with acetic anhydride. The loading yield of the resin was determined using an Fmoc-cleavage UV-assay, and was typically found to be higher than 90%. Subsequent amide couplings were performed in an identical way as in the first route. (Table I, **3**, **4**).

In either synthetic pathway the last three amino acid couplings (Val, Thr and Lys) were troublesome if one of the preceding amino acids had been coupled overnight. Coupling times dramatically increased and refreshing of the amino acid and coupling reagents was found to be necessary. Furthermore, deletions occurred even if the Kaiser test<sup>34,35</sup> indicated complete coupling of the amino acids. A possible cause could be incomplete Fmoc-removal, but increased treatment with piperidine in DMF did not completely prevent the problems. Probably, there is some interaction or folding of the peptide on the resin hampering peptide chain extension. A trivial solution was found in synthesizing the peptide without any interruptions, as diligence has more often proven to enhance the quality of the product of a solid phase peptide synthesis.<sup>36</sup> The combination of the two routes gave full access to independent functionalization of the C- and N-terminus, while keeping the advantages of solid phase peptide synthesis.



**Figure 1.** Solid phase synthesis of N-terminally functionalized peptides **1** ( $n=1$ ), **2** ( $n=15$ ) and **5-9** ( $n=5, 7, 9, 11, 13$  respectively). Reaction conditions: i) piperidine (20% in DMF) ii) Fmoc-Xxx, DIPCDI, HOBt in DMF, iii)  $C_nH_{2n+1}COOH$ , DIPCDI, HOBt in dichloromethane; or acetic anhydride, pyridine in DMF, iv) TFA/H<sub>2</sub>O (95/5)



**Figure 2.** Solid phase synthesis of C-terminally functionalized peptides **3** ( $n=1$ ) and **4** ( $n=15$ ). Reaction conditions: i)  $\text{C}_{16}\text{H}_{33}\text{NH}_2$ , acetic acid,  $\text{NaCNBH}_3$  in DMF/MeOH (1/1), 80 °C ii) Fmoc-Glu(tBu), DIPCPI, HOBT in DMF 1 hour, followed by Fmoc-Glu(tBu), DIPCPI, HOBT in DMF 18 hours, iii) piperidine (20% in DMF), iv) Fmoc-Xxx, DIPCPI, HOBT in DMF, v)  $\text{C}_n\text{H}_{2n+1}\text{COOH}$ , DIPCPI, HOBT in dichloromethane; or acetic anhydride, pyridine in DMF, vi) TFA/ $\text{H}_2\text{O}$  (95/5)

**Table I:** Overview of the peptides synthesized

Peptide	$\text{R}_1$	$\text{R}_2$	Yield (%)
1	$\text{CH}_3$	H	65
2	$\text{C}_{15}\text{H}_{31}$	H	55
3	$\text{CH}_3$	$\text{C}_{16}\text{H}_{33}$	50
4	$\text{C}_{15}\text{H}_{31}$	$\text{C}_{16}\text{H}_{33}$	55
5	$\text{C}_5\text{H}_{11}$	H	75
6	$\text{C}_7\text{H}_{15}$	H	75
7	$\text{C}_9\text{H}_{19}$	H	65
8	$\text{C}_{11}\text{H}_{23}$	H	70
9	$\text{C}_{13}\text{H}_{27}$	H	60

## 2.2.2 Peptide fibril formation

The synthesized KTVIIE-derived peptides were allowed to form fibrils according to a procedure adapted from Serrano *et al.*<sup>21</sup> Each peptide was dissolved in a 20 mM glycine-HCl buffer (pH 2.5) to a concentration of 1 mg mL<sup>-1</sup>. The samples were sonicated at 50 °C for 30 minutes. Some of the amphiphilic peptides did not completely dissolve and were therefore filtered. Subsequently, the samples were left to stand for five days at room temperature. Because the solutions were filtered an assay was necessary to determine the concentration of the peptide. In contrast to the amphiphilic peptides the basic Ac-KTVIIE-NH<sub>2</sub> yielded a clear solution after sonication. Therefore, from this peptide a dilution series was prepared and the absorption of the samples at 220 nm was used to construct a calibration curve, which allowed the determination of the concentrations of the peptide solutions. The peptide fibril solutions could be further diluted to prepare solutions of lower concentrations.

Solutions were prepared in standard Eppendorf tubes, but the determined concentrations of the peptide in the solutions were regularly much lower than expected, even taking into account some losses from the filtration step. Furthermore, the concentration decreased over time even though no precipitation was observed. Probably adherence of the peptides or the fibrils to the standard Eppendorf tube surface was the cause of the losses.<sup>37,38</sup> Therefore, samples were prepared in low adhesion Eppendorf tubes<sup>39</sup> or sealable glass vials, which resulted in peptide concentrations which remained unaltered.

## 2.3 Fibril characteristics

### 2.3.1 Influence of a hydrophobic domain on structure

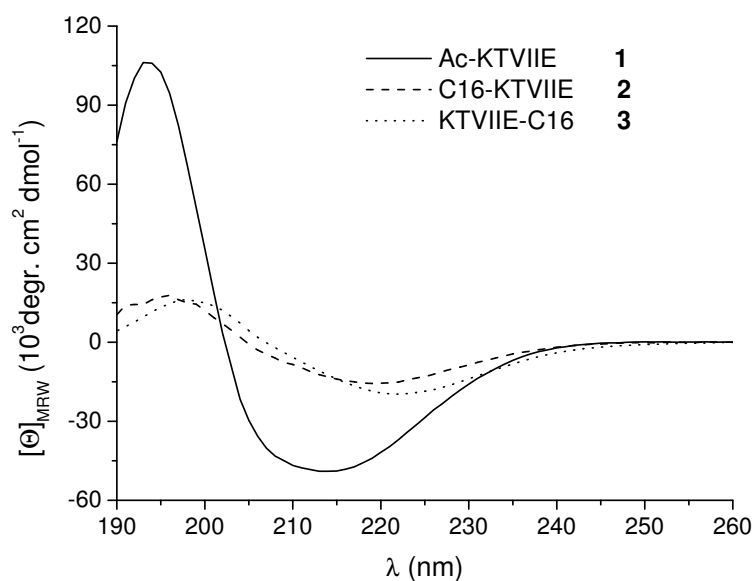
The first molecular assembly parameter that was investigated was the effect of the position of the hydrophobic tail on stabilization of the fibrils. Alkyl tails were therefore introduced in three different ways: at the N-terminus, the C terminus, or both termini were functionalized simultaneously (peptides **2**, **3** and **4** respectively). Alkyl chains containing 16 carbons were chosen as a representative hydrophobic group. Thus, hexadecanoic acid was introduced at the N-terminus or hexadecylamine at the C-terminus, or both were coupled to the same peptide. The aggregation properties of the model peptide KTVIIE **1**<sup>21</sup> were subsequently compared to the properties of these three amphiphilic peptides. In the assembly

process as described by Serrano *et al.*<sup>21</sup> the charged lysine and glutamic acid residues in the peptide direct the aggregation process. The aggregation of the peptide proceeds in two stages, and these stages can be followed using CD spectroscopy. Freshly dissolved, the peptide has a random coil conformation characterized by a minimum near 200 nm. During the assembly process first the dissolved peptide organizes in a  $\beta$ -sheet polymer and a typical  $\beta$ -sheet spectrum develops with a minimum at 220 nm and a maximum at 200 nm. Secondly these polymers aggregate in regular fibril structures.

After filtration, peptide **1** and the singly functionalized peptides **2** and **3** were found to be present in solution at a concentration of 0.6 mM. In contrast, peptide **4** with two introduced hydrophobic tails proved to be highly insoluble. No UV-Vis or Circular Dichroism (CD) spectra could be recorded and no fibrils could be found using Transmission Electron Microscopy (TEM). Apparently, the two hydrophobic tails decreased the solubility of peptide **4** too much and structured aggregates were not formed anymore, therefore this peptide was not examined any further.

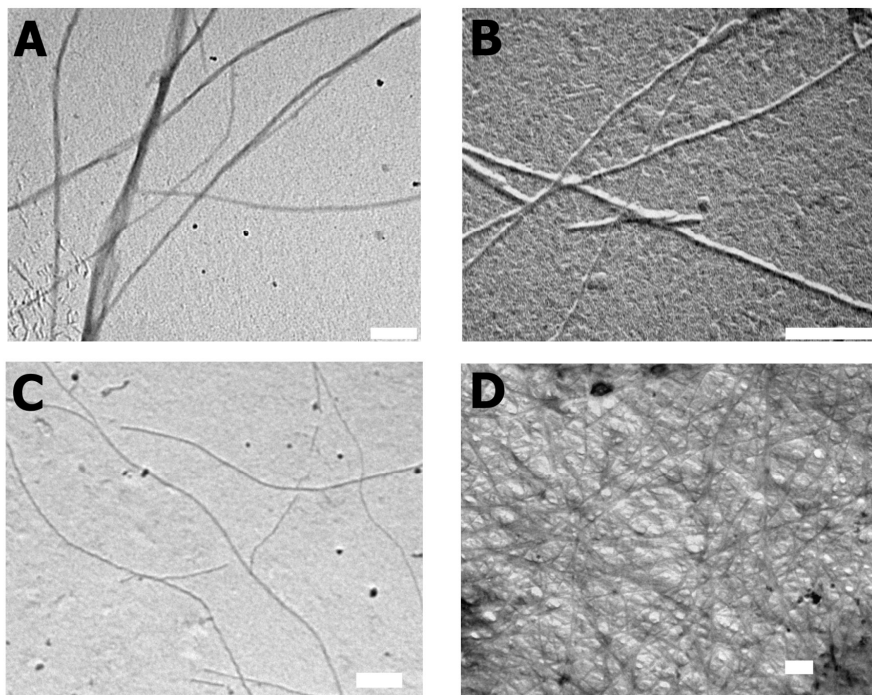
The CD spectrum of model peptide **1** showed a negative signal at 220 nm and a positive peak at 200 nm (Figure 3). This spectrum is typical for a  $\beta$ -sheet type assembly and is in accordance with previous reports.<sup>21</sup> Similarly, the CD spectra of solutions of both singly modified peptides **2** and **3** showed a minimum near 220 nm and a maximum at 200 nm.

The high molar ellipticity of peptide **1** was attributed to a contribution of linear dichroism.<sup>40,41</sup> The position of the minimum near 220 nm of the N- and C- terminally modified peptides differed slightly between the two peptides. This shift probably originates from small deviations of the actual peptide folding from a perfect  $\beta$ -sheet. A typical CD signal for a  $\beta$ -sheet has been determined using large proteins; in the small peptide contributions from end groups that fold slightly differently will contribute significantly to the final CD spectrum.



**Figure 3.** CD spectra of 0.6 mM solutions of amyloid fibril forming peptides **1-3**. The curves are typical for a  $\beta$ -sheet conformation of the peptides.

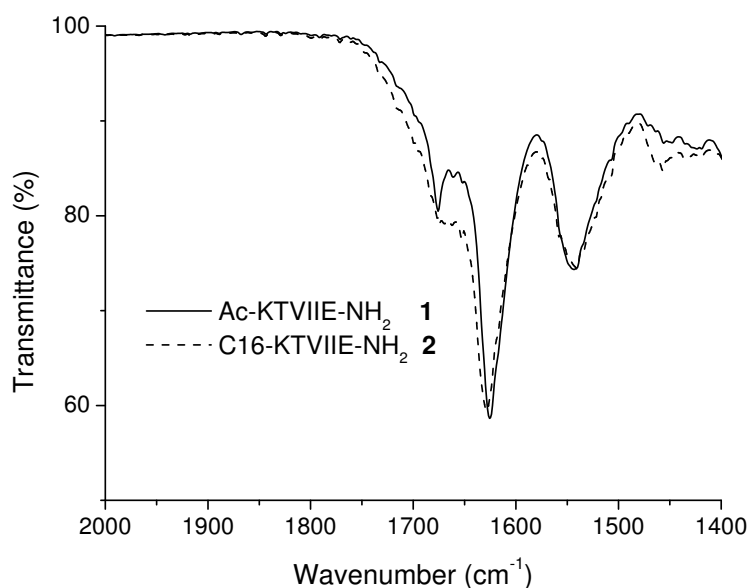
Furthermore, TEM pictures showed in all cases the presence of fibrils (Figure 4); fibrils obtained from the alkylated peptides possessed a similar morphology as the fibrils formed by the non-functionalized peptide. Both twisted and straight fibrils could be observed in TEM pictures of all samples, and fibrils had assembled into larger aggregates. Typically, fibrils were several micrometers long. Upon increasing the exposure time of a carbon coated TEM grid to a fibril solution the fibril density increased, until the fibril density became too high to discern individual structures, which is indicative of their inclination to stick to hydrophobic surfaces.



**Figure 4.** TEM pictures of Ac-KTVIIE **1** (a), C16-KTVIIE **2** (b), Ac-KTVIIE-C16 **3** (c) and of C16-KTVIIE **2** with high concentration of fibrils on grid (d); bars represents 200 nm

To further probe the internal structure of the fibers infrared spectra were recorded of lyophilized fibrils<sup>42</sup> from aqueous solutions of peptides **1**, **2** and **3**. The amide I and II bands are indicative of the folding of peptides in aggregates.<sup>43,44</sup> All samples showed a strong amide I band at  $1625\text{ cm}^{-1}$  and the amide II band at  $1535\text{ cm}^{-1}$  consistent with a  $\beta$ -sheet conformation of the peptides (figure 5, table II). The presence of alkyl tails was evident from the corresponding absorption band of the C-H vibration at  $2925$  and  $2850\text{ cm}^{-1}$ .





**Figure 5.** Amide vibrations of peptides **1**, **2**.

**Table II:** Observed frequencies (cm<sup>-1</sup>) of polypeptides in various conformations<sup>44</sup> compared to the frequencies observed for lyophilized samples of fibrils.

	Amide I		Amide II	
Random coil	1656 (s)		1535 (s)	
Alpha helix	1650 (s)	1652 (m)	1516 (w)	1546 (s)
Parallel beta	1645 (w)	1630 (s)	1530 (s)	1550 (w)
Anti Parallel beta	1685 (w)	1632 (s)	1530 (s)	
Ac-KTVIIE <b>1</b>	1675 (w)	1625 (s)	1545 (s)	
C16-KTVIIE <b>2</b>	1675 (w)	1625 (s)	1545 (s)	
Ac-KTVIIE-C16 <b>3</b>	1675 (w)	1625 (s)	1545 (s)	

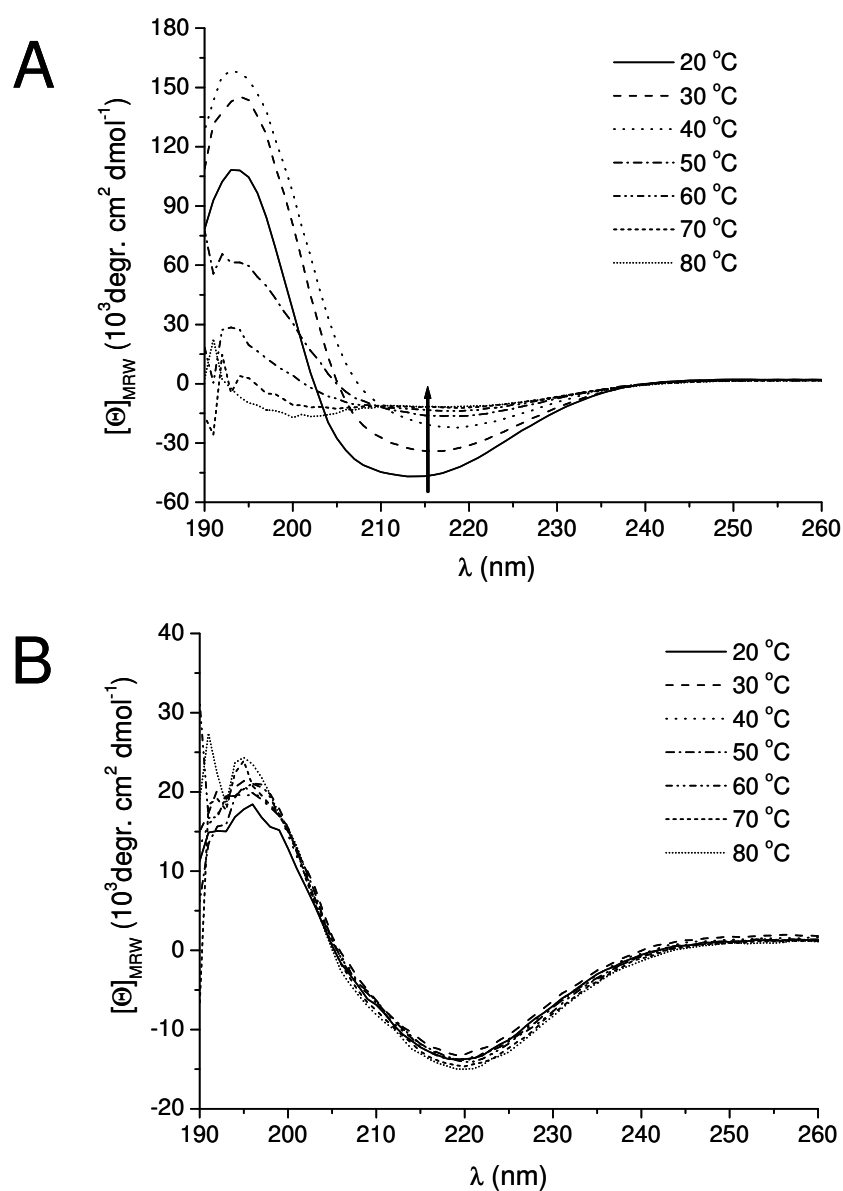
These IR data indicate that the peptide conformation and the fibril structure are independent on the presence of the alkyl tails. The lyophilization procedure could possibly have changed the conformation of the peptide, but the fibrils are already present in solution, and correctly folded enzymes can be lyophilized without losing activity. Furthermore,  $\beta$ -amyloid aggregates are structurally much more stable than proteins; therefore, the fibrils and the peptide conformation are expected to remain intact during lyophilization.

It can therefore be concluded that peptide assembly and conformation is not affected by the introduction of the hydrophobic tails, which furthermore did not seem to promote non-specific aggregation of the amphiphilic peptides.

### 2.3.2 Temperature and concentration dependence

As described above, introduction of a hydrophobic tail to either terminus of hexapeptide KTVIIE did not affect the tendency of the peptide to form fibrils in solution, nor its conformation and fiber morphology. Temperature-dependent CD measurements were deployed to investigate whether the stability of the fibril structures was affected by the presence of the alkyl chains.<sup>45</sup> On increasing the temperature to 80 °C the spectrum of the model peptide **1** changed to a minimum at 200 nm and a maximum at 220 nm (Figure 6). Such a spectrum is typical for a random coil-conformation of this peptide. This change was not readily reversible upon cooling, indicating that fibril structures were completely disrupted. The  $\beta$ -sheet spectrum returned only slowly after maintaining the peptide solution for several days at room temperature.

In contrast, the CD spectra of both the C- and N-terminally modified peptides **2** and **3** did not change on increasing the temperature up to 90 °C (Figure 6), neither did subsequent cooling of the samples lead to any change in structure. TEM showed that fibrils were still present in the solutions of the amphiphilic peptides after heating the sample, in accordance with the observed CD-signal. The temperature experiments show that the properties of the peptide fibrils are not only determined by the amino acids, but also by the hydrophobic tail. The hydrophobic tails introduce stability to the peptide fibrils, to the extent that the peptide structure stays unchanged over a temperature range from 4 °C up to 90 °C.

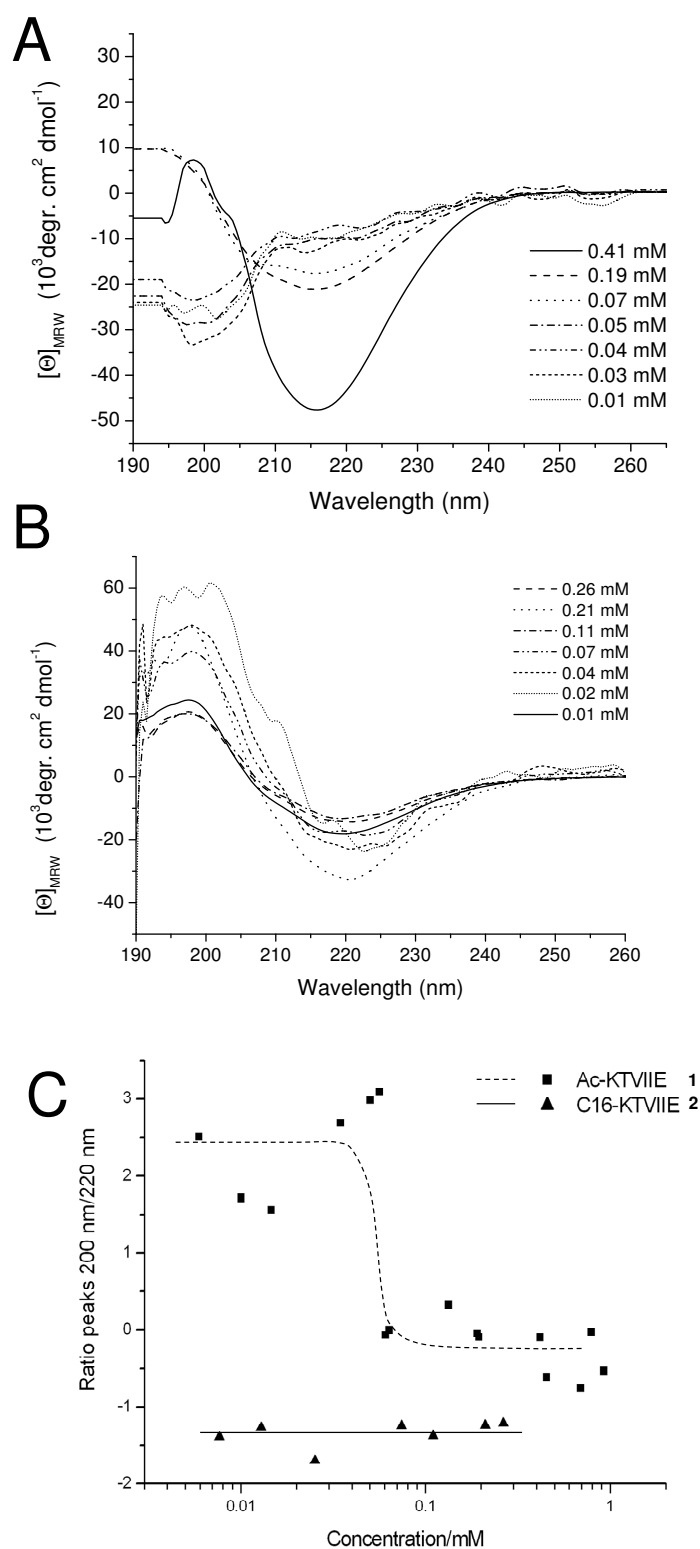


**Figure 6.** CD spectra of peptides in the range of 20-80 °C; A) Ac-KTVIIE 1, arrow indicates the change on temperature increase from a spectrum indicating a  $\beta$ -sheet to a random conformation; B) C16-KTVIIE 2, showing a spectrum typical for a  $\beta$ -sheet at all temperatures.

As a second tool to probe the stability of the fibrils, the effect of peptide concentration on assembly behavior was investigated for the series of peptide **1** and **2**. A solution of an amphiphile will contain assemblies if the concentration is higher than the critical aggregation concentration, below which the amphiphile will be molecularly dissolved. The higher hydrophobicity of the amphiphilic peptides should lower this critical concentration compared to that of Ac-KTVIIIE-NH<sub>2</sub>. Fibrils formed by peptide **2** were therefore expected to be stable at a lower concentration.

A dilution series was made with glycine buffer, starting from a peptide concentration of approximately 0.6 mM which showed a minimum at 220 nm and a maximum at 200 nm in the CD spectrum. The diluted samples were equilibrated overnight at room temperature. The CD spectra were recorded in a 1 mm quartz cell, since the absorption of the used glycine buffer below 200 nm prohibited the use of a 1 cm cell.

Diluting the solution of peptide **1** caused a change of the CD-spectrum. At concentrations below 60  $\mu$ M the minimum at 220 nm in the CD spectrum disappeared and a minimum at 195 nm appeared. This indicates that also upon dilution the fibrils disassemble. In contrast, the CD spectrum of the amphiphilic peptide **2** did not change upon dilution; the minimum at 220 nm was present down to the lowest concentrations in which the CD signal could be measured accurately (Figure 7). Therefore, introduction of the hydrophobic tail to the peptide sequence stabilizes the fibrils formed by the peptide down to a concentration of at least 10  $\mu$ M.



**Figure 7.** CD spectra of fibril forming peptide at a concentration range. A) Ac-KTVIIE 1 showing a transition below 7  $\mu\text{M}$ ; B) C16-KTVIIE 2 showing a constant signal at all concentrations; C) Ratio of the peaks at 200 nm to 220 nm of peptides 1 and 2 at the concentrations. Lines are presented to guide the eye.

CD spectroscopy, TEM and IR spectroscopy indicated that the introduction of an alkyl tail did not influence fibril structure and the folding of the peptide in the fibril. In order to determine whether the peptides **1** and **2** would co-assemble the mixing behavior of the hexapeptides was examined in some preliminary experiments. Clear aqueous solutions of fibrils of Ac-KTVIIE-NH<sub>2</sub> and C16-KTVIIE-NH<sub>2</sub> were mixed in a 1:1 molar ratio. The mixture was sonicated for 60 minutes at 50 °C and left overnight at room temperature. Temperature scan measurements in the CD spectrometer showed characteristics of both stable C16-KTVIIE-NH<sub>2</sub> assemblies and the dissolution of Ac-KTVIIE-NH<sub>2</sub> assemblies. CD spectra recorded after cooling down the samples also indicated that separate assemblies of both peptides were present. Therefore, with this experimental setup peptides **1** and **2** do not co-assemble into fibrils.

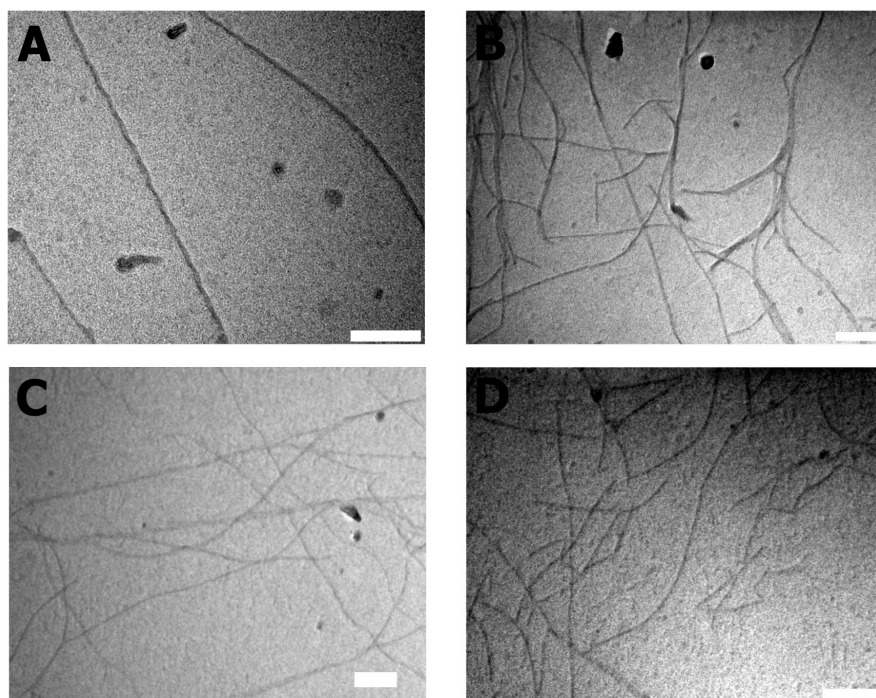
In conclusion, introduction of a hydrophobic alkyl tail to the KTVIIE peptide sequence increases the stability of the fibrils formed by the peptide. At the same time the structure of the fibrils is not influenced by the introduction of the alkyl tail.

## **2.4 Varying the hydrophobic domain**

### **2.4.1 Influence of chain length on peptide assembly**

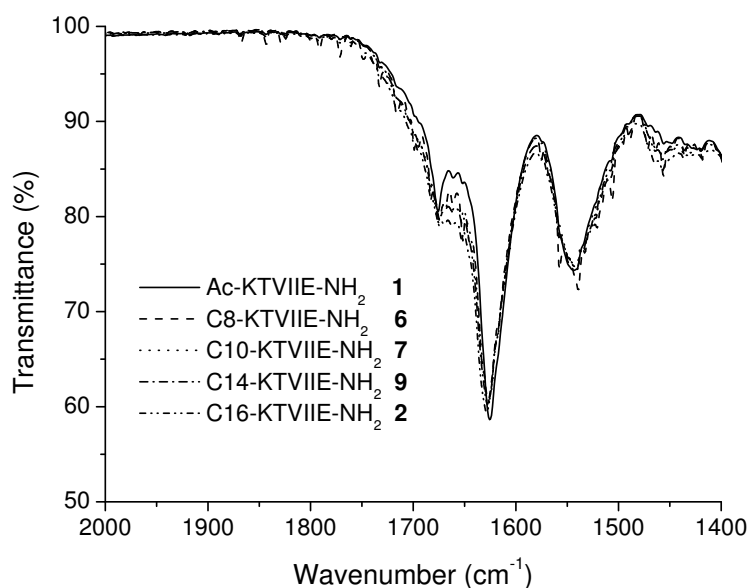
To further examine the scope of the stabilization a series of peptides was synthesized with decreasing hydrophobic character. In phospholipids the length of the hydrophobic tails influences the assembly properties of the molecules. The longer the hydrophobic groups the lower the critical assembly concentration. Furthermore, the length of the alkyl group influences other properties such as melting temperature of the assemblies. Therefore, variation of the alkyl tail length in the lipopeptides could be used to fine-tune the assembly properties.

Since C-terminally and N-terminally functionalized peptides showed identical stabilization, only the N-functionalized series was prepared, because the latter are synthetically more readily available. The aggregation properties of peptides **5-9** at room temperature and at a concentration of 0.5 mg mL<sup>-1</sup> were first examined. The CD spectra of these peptides showed a negative peak at 220 nm and a positive peak at 200 nm indicating the presence of peptide aggregates of a structure similar to peptides **1** and **2**, TEM pictures again showed the presence of fibrils with a morphology comparable to that of the model peptide (figure 8).



**Figure 8.** TEM pictures of C8-KTVIIE **6** (a), C10-KTVIIE **7** (b), C12-KTVIIE **8** (c) and of C14-KTVIIE **9** (d); (bars represents 200 nm)

IR spectra of the peptides showed a strong amide I band at  $1625\text{ cm}^{-1}$  and the amide II band at  $1535\text{ cm}^{-1}$  consistent with a  $\beta$ -sheet conformation (figure 9, table III). The increasing length of the alkyl tails was reflected in a corresponding increase in the absorption band of the C-H vibration at  $2925$  and  $2850\text{ cm}^{-1}$ . Again this was in accordance with the structures of both peptide **1** and **2** as described above. Varying the hydrophobic domain from the acetyl to the C16 tail does not influence the assembly behavior and conformation of the peptide, as far as can be observed by IR spectroscopy.



**Figure 9.** IR spectra of hydrophobically modified KTVIIE peptides in the amide vibration region.

**Table III:** Observed frequencies ( $\text{cm}^{-1}$ ) of polypeptides in various conformations<sup>44</sup> compared to the frequencies observed for lyophilized samples of fibrils.

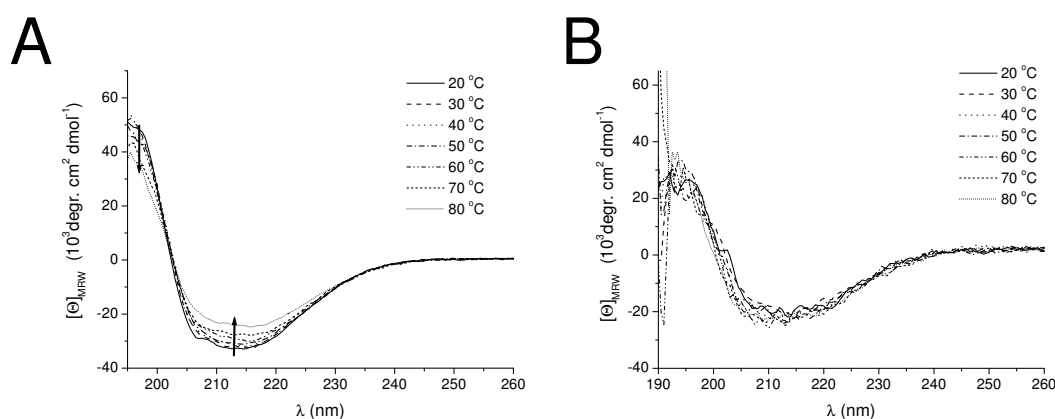
	Amide I		Amide II	
Random coil	1656 (s)		1535 (s)	
Alpha helix	1650 (s)	1652 (m)	1516 (w)	1546 (s)
Parallel beta	1645 (w)	1630 (s)	1530 (s)	1550 (w)
Anti Parallel beta	1685 (w)	1632 (s)	1530 (s)	
C8-KTVIIE <b>6</b>	1675 (w)	1625 (s)	1545 (s)	
C10-KTVIIE <b>7</b>	1675 (w)	1625 (s)	1545 (s)	
C14-KTVIIE <b>9</b>	1675 (w)	1625 (s)	1545 (s)	

### 2.4.2 Temperature and concentration dependence

The influence of length of the hydrophobic group on temperature stability of the peptide fibrils was examined.<sup>46</sup> On increasing the temperature of a 0.6 mM solution of the C6 functionalized peptide **5** the CD spectrum showed a marked decrease of the intensity of the peak at 220 nm. Keeping the sample at 80 °C the peak at 220 nm disappeared whereas a peak at 190 nm became more intense, indicating a decrease in structure and hence the disassembly of the fibrils. Upon cooling, the peak at 220 nm did not return. Compared to model peptide **1** the disappearance of the fibrils is however a slower process. The transition temperature of peptide **5** was 70 °C, whereas in case of the non-modified peptide **1** already at 40 °C the process of disassembly could be observed.

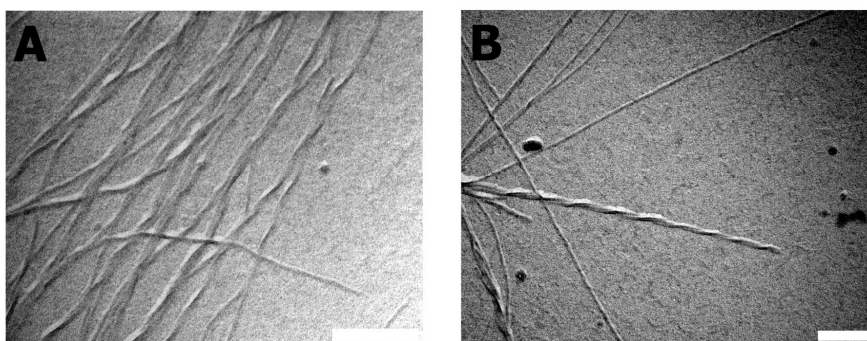


The CD spectra of the C8 and C10 functionalized peptides **6** and **7** also changed upon heating. The intensity of the minimum at 220 nm and the maximum at 200 nm decreased (figure 10). However, the CD-spectra did not change into spectra which were reminiscent of random coil structures. Furthermore, the change was fully reversible; upon cooling the original CD-spectra were obtained. The solution of C12 functionalized peptide **8** showed a stable CD spectrum in the whole examined temperature range. Expectedly peptide **9** with the C14 tail also had a stable CD spectrum already observed for the C16 functionalized peptides **2** and **3** (figure 10). TEM pictures of the solutions of the peptides with alkyl tails of C8 (**6**) and longer showed fibrils, irrespective of thermal history (Figure 11).



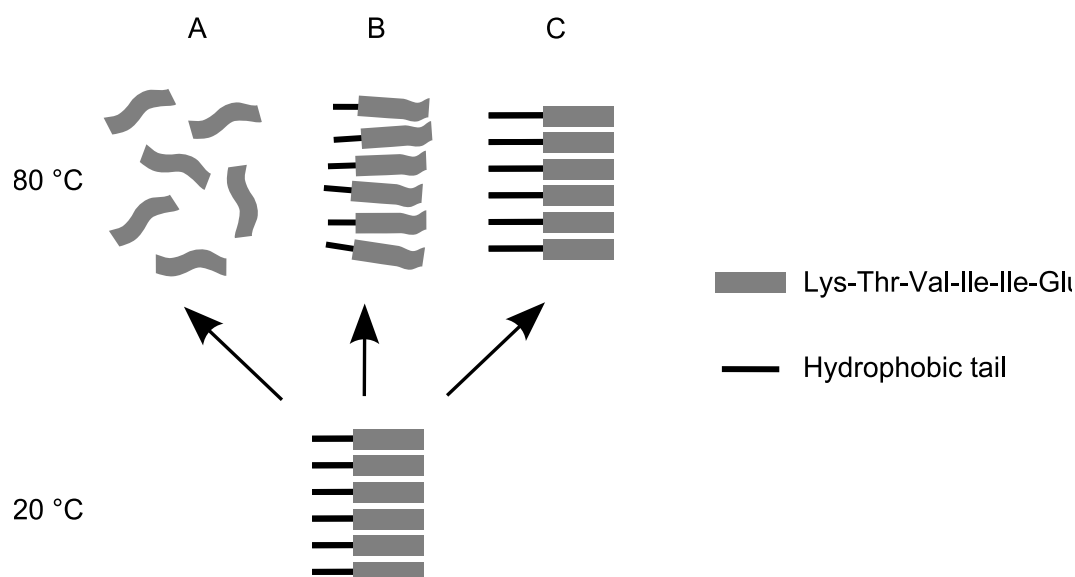
**Figure 10.** CD spectra at different temperatures in the range from 20-80 °C of C8-KTVIIE **6** (left) and C12-KTVIIE **8** (right). Arrow indicates the change on temperature increase

The temperature measurements show that a small increase in hydrophobic interactions can already have a profound effect on the stability of peptide aggregates and peptide folding in the aggregate. Upon the introduction of the C6 alkyl tail the disassembly temperature increases by 30 °C compared to the fibers obtained from the non-alkylated peptide **1**. However, the general temperature behavior of the molecule is still the same and the fibrils completely dissociate while the formation of the fibrils is seemingly not affected. Increasing the alkyl length to C8 or C10 moves the disassembly temperature so high it is not attainable anymore in water.



**Figure 11.** TEM pictures of fibrils formed by C8-KTVIIE **6** before (A) and after (B) heating to 80 °C (bars represents 200 nm).

For peptides **6** and **7** with a C8 and C12 tail respectively the CD signal changed somewhat on increasing the temperature. This change was readily reversible suggesting the assemblies were at least partly intact, though the peptide conformation changed slightly. The change in the CD spectrum suggests that while the fibrils are stable at increased temperatures the peptide structure is flexible (Figure 12). Finally, extending the alkyl tail length to 12 and higher to 14 and 16 carbon atoms, the conformation of the peptide is fixed and the assembly is stable.



**Figure 12.** Effect of hydrophobic tail on peptide fibril stability at 80 °C. A) Soluble peptides when the tail is C6 or shorter, B) Some freedom for conformational change of the peptide in the fibril for C8 and C10, C) Stable fibrils at 80 °C for C12 and longer hydrophobic tails.

Because a marked difference was found for peptides **1** and **2** in their behavior upon dilution also the concentration dependence of aggregates of acylated peptides **5-9** was determined using CD spectroscopy. On dilution the CD spectrum of the C6 functionalized peptide **5** changed at concentrations below 40  $\mu\text{M}$ . Compared to model peptide **1** the transition took place over a broader concentration range, and at a lower concentration. Similarly to the effect on the aggregate stability at increased temperatures the hexyl tail was capable to a certain level of stabilization.

In contrast to peptide **5** with the C6 tail the aggregates of peptides with longer tails did not disassemble upon dilution. The CD spectrum showed a distinct minimum at 220 nm down to the lowest concentration that could be measured, indicating that the peptide fibrils are stable at low micromolar concentrations. No difference was observed between the CD spectra of C8 compound **6** and C12 compound **8** in these dilution series. This is in contrast to the difference observed in the CD spectra of these compounds at increased temperatures described above. This suggests that the behavior at increased temperature is related to the melting temperature of the n-alkyl chains.

In conclusion introduction of an alkyl tail to the KTVIIE peptide stabilizes the assemblies formed. Tails shorter than C6 induce some stabilization but the fibrils disassemble at 80 °C and below 40  $\mu\text{M}$ . Introduction of C8 and C10 chains results in stable fibrils, although there is some flexibility in the assemblies at 80 °C and the folding of the peptide can change somewhat. For C12 and longer hydrophobic tails the conformation of the peptide in the fibril is stabilized to 90 °C.

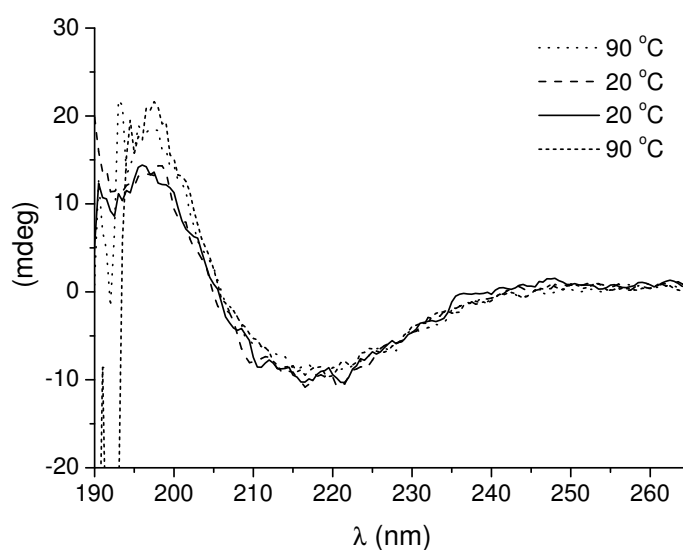
## **2.5 Fluorous stabilization**

Not only normal alkyl groups can be used to introduce hydrophobicity, also fluorinated alkyl chains can be used for this purpose. Fluorinated alkyl tails not only behave hydrophobically in aqueous environment, but can also form assemblies in organic solvents in which alkanes dissolve.<sup>47</sup> Furthermore, fluorinated amino acids have been introduced in sequences to enhance selectivity of peptide interactions and folding.<sup>48-50</sup>

Fluorous tails were introduced at the N-terminus of the Lys-Thr-Val-Ile-Ile-Glu hexapeptide. As described above the hexapeptide was synthesized on the solid phase. Two different fluorinated tails were introduced using  $\text{C}_6\text{F}_{13}\text{C}_2\text{H}_2\text{COOH}$  and  $\text{C}_7\text{F}_{15}\text{COCl}$ . The former was synthesized following a literature procedure.<sup>51</sup> A standard peptide coupling using DIPCDI as a coupling agent in the presence of HOBt in DMF was used to couple

$C_6F_{13}C_2H_2COOH$  to the peptide N terminus. The acid chloride  $C_7F_{15}COCl$  was added to the peptide in the presence of DIPEA. This approach resulted in complete acylation of the N-terminus of the peptide. The fluorinated peptides were cleaved off the resin using TFA/H<sub>2</sub>O (95/5).

The peptides resulted in clear solutions in MilliQ water at a concentration of 1 mg/mL, after sonication at 50 °C. The CD spectra of these solutions showed a  $\beta$ -sheet like spectrum with a minimum at 220 and a maximum at 200 nm. Upon heating the sample from 20 °C to 90 °C and cooling back again there was a reversible increase of the intensity maximum at 200 nm. This change in the CD spectrum is opposite to the change observed for the alkylated peptides (**6** and **7**, with C8 and C10 tails respectively), where the peak at 200 decreased in intensity upon an increase in temperature. The difference might be a result of a different aggregation behavior of the fluorinated alkyl tails compared to the standard tails. Still, the stabilizing effect of the fluorous tail is apparent.



**Figure 13.** CD spectra of  $C_7F_{15}CO-KTVIIE-NH_2$  at 20 and 90 °C

$C_7F_{15}CO-KTVIIE-NH_2$  was mixed with Ac-KTVIIE- $NH_2$  in a 1:1 ratio. The peptides were separately dissolved in trifluorethanol, the solutions were mixed and after evaporation to dryness redissolved in MilliQ to a final concentration of 1 mg mL<sup>-1</sup>. Solutions of the pure compounds were prepared following the identical procedure. The CD spectra of pure compounds were identical to the spectra of the same peptides directly dissolved from solid. The spectrum of the mixture was consistent with a linear combination of the spectra of both compounds in the mixture.

Introduction of a fluorinated tail to the KTVIIE peptide stabilized the assemblies of the peptide. The stabilization was similar to the effect of an alkyl tail of the same length, showing stabilization of the fibrils at increased temperatures with a small and reversible change in conformation of the peptide.

## 2.7 Conclusion

Based on the results described in this chapter it can be concluded that introduction of hydrophobic tails to peptide **1** is an efficient method to stabilize aggregates formed by this peptide in glycine buffer. Remarkably TEM, IR and CD spectroscopy show that the morphology of the aggregates and the conformation of the peptide are not influenced by the introduction of the additional hydrophobic interactions. This strong morphological stability is independent of functionalization at either N- or C-terminus of the peptide. The peptide therefore controls the morphology of the aggregates, while introduction of alkyl tails only results in a higher stability of the fibrils to an increased temperature and lower concentrations. Although the introduction of linear hydrocarbons with up to 10 carbon atoms already leads to remarkable added thermal stability, this induced stability seems to be maximal when the alkyl tail is C12 or longer. In contrast to previous examples where the systematic increase of hydrophobic interactions showed a steady increase in melting temperature<sup>52</sup> or a slow change in aggregate morphologies,<sup>7</sup> in our  $\beta$ -sheet forming peptide added hydrophobic interactions seem to work as a switch without affecting the peptide conformation.

## 2.8 Experimental

### Methods and materials

<sup>1</sup>H-NMR spectra were measured on a Varian Inova 400 MHz NMR spectrometer. MALDI-TOF spectra were measured on a Bruker Biflex II mass spectrometer, 2,5-dihydroxybenzoic acid (DHB) was used as matrix. UV-Vis measurements were performed on a Varian Cary 50 UV-Vis spectrophotometer and CD measurements were performed on a Jasco J-810 spectropolarimeter with Peltier temperature control. IR spectra were recorded on a Thermo Wattson IR300 spectrometer, fitted with a Harrick ATR unit. Transmission Electron Microscopy was performed on a JEOL 1010 transmission microscope. Peptides were synthesized on a Labortec SP4000 or a Labortec SP640 semi-automatic peptide synthesizer. Peptide couplings were followed to completion using the Kaiser test.<sup>34</sup> Low adhesion eppendorf tubes were obtained from BIOplastics (B74030). All reagents were obtained from common commercial sources and used as received.

### Synthesis of peptide R<sub>1</sub>-Lys-Thr-Val-Ile-Ile-Glu-NH<sub>2</sub> (1,2,5-9)

The peptide was synthesized using standard Fmoc peptide synthesis on a Breipohl resin.<sup>27,28</sup> Peptide coupling was achieved using 3 equivalents of Fmoc amino acid and diisopropylcarbodiimide (DIPCDI, 3.3 eq.) with N-hydroxy benzotriazole (HOBt, 3.6 eq.) in DMF. The resin was swollen in DMF for 20 minutes prior to use. The Fmoc-group was removed using piperidine in DMF (20% v/v, 3 times 6 minutes). After coupling of the

final amino acid the Fmoc-protected peptide on the resin was washed thoroughly with DMF, dichloromethane, methanol and diethyl ether. The resin was dried in vacuum and divided in batches.

The dry resin was swollen for 30 minutes in DMF. Subsequently the Fmoc protecting group was removed using piperidine in DMF (20% v/v). The resin was washed well with DMF and dichloromethane. Three equivalents of the aliphatic carboxylic acid were dissolved in dichloromethane and 3.6 equivalents of HOBt (1M in DMF) were added. After mixing 3.3 equivalents of neat DIPCDI were added. The solution was added to the resin and the resin was agitated for 18 hours. Peptide **1** was synthesized by treating the detracted peptide on the resin with acetic anhydride. To DMF 10 equivalents of acetic anhydride and 20 equivalents of pyridine was added. This mixture was added to the resin and the mixture was agitated for 30 minutes. After washing the resin with DMF, dichloromethane, methanol and diethyl ether the resin was dried in vacuo. The peptides were cleaved from the resin by treatment with trifluoroacetic acid/water (95/5, v/v) for 4 hours. After precipitation in diethyl ether the peptides were lyophilized from acetic acid. The peptides were characterized using <sup>1</sup>H-NMR spectroscopy and MALDI-TOF mass spectrometry of which the details are summarized in the table below.

### Synthesis of peptide R1-Lys-Thr-Val-Ile-Ile-Glu-NHC<sub>16</sub>H<sub>33</sub> (3,4)

The C-terminal n-hexadecylamine tail for peptides **3** and **4** was introduced through reductive amination of 4-(4-formyl-3-methoxyphenoxy)butyl aminomethyl resin.<sup>53</sup> In 15 mL of a mixture of DMF and methanol (1/1, v/v) 10 mmol of n-hexadecylamine was dissolved. To this solution 10 mmol of acetic acid, 10 mmol of NaCNBH<sub>3</sub> and 1.0 g of resin (1.0 mmol) were added. The mixture was stirred slowly and heated to 80 °C for two hours. Subsequently the resin was washed thoroughly with methanol and dichloromethane. To the secondary amine formed on the resin Fmoc protected glutamic acid was coupled using standard Fmoc solid phase methodology using 3 equivalents of the protected amino acid dissolved in DMF, HOBt (1M in DMF, 3.6 eq) and DIPCDI (1M in DMF, 3.3 eq.). After 1 hour this reaction mixture was refreshed and subsequently the reaction mixture was left for 18 hours. The resin was washed with DMF and treated with 10 equivalents of acetic anhydride in the presence of pyridine to cap any possibly remaining secondary amines. The resin was washed using DMF, methanol, dichloromethane and diethyl ether and was dried thoroughly. The loading of the dry resin was determined using an Fmoc-cleavage UV-assay and was found to be 0.37 mmol g<sup>-1</sup> (75 % of theoretical yield).

With the resulting resin the other amino acids were again coupled using the standard Fmoc solid phase synthesis protocol. After coupling of the final amino acid, the Fmoc-protected peptide on the resin was washed thoroughly with DMF, dichloromethane, methanol and diethyl ether. The resin was dried in vacuum and divided in batches. The dry resin was swollen for 30 minutes in DMF. Subsequently the Fmoc protecting group was removed using piperidine in DMF (20% v/v). The resin was washed well with DMF and dichloromethane. Acylation of the N-terminus was achieved by either a standard peptide coupling of n-hexanoic acid or treatment with acetic anhydride in the presence of pyridine. In the first case (**4**) 3 equivalents of n-hexanoic acid was dissolved in dichloromethane and 3.6 equivalents of HOBt (1M in DMF) were added. Finally 3.3 equivalents of neat DIPCDI was added to this solution. The resin was washed using DMF, dichloromethane, methanol and diethyl ether. After drying the products were cleaved from the resin by treatment with trifluoroacetic acid (95%, water 5%) during three hours. After cleavage the peptides were purified by precipitation in diethyl ether. The peptides were characterized using <sup>1</sup>H-NMR spectroscopy and MALDI-TOF mass spectrometry

MALDI-TOF mass spectra peptides **1-9**

Compound	Structural formula	(M+H) <sup>+</sup> calc	(M+Na) <sup>+</sup> calc	(M+H) <sup>+</sup>	(M+Na) <sup>+</sup>
<b>1</b>	C <sub>34</sub> H <sub>62</sub> N <sub>8</sub> O <sub>10</sub>	743.46	765.45	743.08	765.07
<b>2</b>	C <sub>48</sub> H <sub>90</sub> N <sub>8</sub> O <sub>10</sub>	939.68	961.67	939.25	961.24
<b>3</b>	C <sub>50</sub> H <sub>94</sub> N <sub>8</sub> O <sub>10</sub>	967.71	989.70	967.33	989.31
<b>4</b>	C <sub>64</sub> H <sub>122</sub> N <sub>8</sub> O <sub>10</sub>	1163.93	1185.92	1163.67	1185.66
<b>5</b>	C <sub>38</sub> H <sub>70</sub> N <sub>8</sub> O <sub>10</sub>	799.52	821.51	799.36	821.38
<b>6</b>	C <sub>40</sub> H <sub>74</sub> N <sub>8</sub> O <sub>10</sub>	827.55	849.54	827.41	849.39
<b>7</b>	C <sub>42</sub> H <sub>78</sub> N <sub>8</sub> O <sub>10</sub>	855.58	877.57	855.48	877.43
<b>8</b>	C <sub>44</sub> H <sub>82</sub> N <sub>8</sub> O <sub>10</sub>	883.62	905.61	883.47	905.50
<b>9</b>	C <sub>46</sub> H <sub>86</sub> N <sub>8</sub> O <sub>10</sub>	912.65	934.64	912.33	934.31

II <sup>1</sup>H-NMR spectra peptides 1-9*CH<sub>3</sub>CO-Lys-Thr-Val-Ile-Ile-Glu-NH<sub>2</sub> 1*

12.0 (br s, 1H), 8.0 (d, 1H), 7.9 (m, 1H), 7.8 (m, 2H), 7.5 (m, 1H), 7.1 (s, 1H), 7.0 (s, 1H), 4.9 (s, 1H), 4.3 (q, 1H), 4.2 (q, 2H), 4.1 (t, 3H), 3.9 (q, 1H), 2.7 (t, 1H), 2.2 (t, 2H), 1.9 (s, 1H), 1.8 (s, 3H), 1.7 (m, 4H), 1.5 (q, 2H), 1.4 (m, 2H), 1.3 (m, 3H), 1.0 (m, 4H), 0.8 (m, 24H)

*C<sub>15</sub>H<sub>31</sub>CO-Lys-Thr-Val-Ile-Ile-Glu-NH<sub>2</sub> 2*

12.0 (br s, 1H), 8.0 (d, 1H), 7.9 (d, 1H), 7.85 (t, 2H), 7.7 (d, 1H), 7.5 (m, 3H), 7.0 (s, 1H), 7.9 (s, 1H), 4.9 (s, 1H), 4.2 (q, 2H), 4.1 (t, 3H), 3.9 (q, 1H), 2.7 (t, 2H), 2.2 (t, 2H), 2.1 (t, 2H), 1.9 (m, 2H), 1.7 (m, 4H), 1.5 (m, 4H), 1.4 (m, 2H), 1.2 (s, 28H), 1.05 (q, 2H), 0.95 (d, 2H), 0.8 (t, 2H), 0.75 (m, 20H)

*CH<sub>3</sub>CO-Lys-Thr-Val-Ile-Ile-Glu-NHC<sub>16</sub>H<sub>33</sub> 3*

12.0 (br s, 1H), 8.0 (d, 1H), 7.9 (d, 1H), 7.85 (t, 2H), 7.75 (d, 1H), 7.7 (t, 1H), 7.6 (m, 3H), 4.8 (d, 1H), 4.3 (q, 1H), 4.2-4.1 (m, 5H), 3.9 (q, 1H), 2.7 (q, 2H), 2.2 (q, 2H), 1.9 (s, 1H), 1.8 (s, 3H), 1.65 (m, 4H), 1.45 (q, 3H), 1.4 (m, 6H), 1.2 (s, 33 H), 1.0 (m, 4H), 0.8 (m, 24H)

*C<sub>15</sub>H<sub>31</sub>CO-Lys-Thr-Val-Ile-Ile-Glu-NHC<sub>16</sub>H<sub>33</sub> 4*

12.0 (br s, 1H), 8.0 (d, 1H), 7.9 (d, 1H), 7.85 (t, 2H), 7.7 (d, 1H), 7.5 (m, 3H), 7.0 (s, 1H), 7.9 (s, 1H), 4.9 (s, 1H), 4.2 (q, 2H), 4.1 (t, 3H), 3.9 (q, 1H), 2.7 (t, 2H), 2.2 (t, 2H), 2.1 (t, 2H), 1.9 (m, 2H), 1.7 (m, 4H), 1.5 (m, 4H), 1.4 (m, 2H), 1.2 (s, 60H), 1.05 (q, 2H), 0.95 (d, 2H), 0.8 (m, 26H)

*C<sub>5</sub>H<sub>9</sub>CO-Lys-Thr-Val-Ile-Ile-Glu-NH<sub>2</sub> 5*

12.0 (br s, 1H), 8.0 (d, 1H), 7.9 (d, 1H), 7.8 (m, 3H), 7.5 (m, 3H), 7.0 (s, 1H), 7.9 (s, 1H), 4.9 (s, 1H), 4.2 (q, 2H), 4.1 (t, 3H), 3.9 (q, 1H), 2.7 (t, 2H), 2.2 (t, 2H), 2.1 (t, 2H), 1.9 (m, 2H), 1.7 (m, 4H), 1.5 (m, 4H), 1.4 (m, 2H), 1.2 (s, 4H), 1.05 (q, 2H), 0.95 (d, 2H), 0.8 (t, 2H), 0.75 (m, 20H)

*C<sub>7</sub>H<sub>15</sub>CO-Lys-Thr-Val-Ile-Ile-Glu-NH<sub>2</sub> 6*

12.0 (br s, 1H), 8.0 (d, 1H), 7.9 (d, 1H), 7.85 (t, 2H), 7.7 (d, 1H), 7.5 (m, 3H), 7.0 (s, 1H), 7.9 (s, 1H), 4.9 (s, 1H), 4.2 (q, 2H), 4.1 (t, 3H), 3.9 (q, 1H), 2.7 (t, 2H), 2.2 (t, 2H), 2.1 (t, 2H), 1.9 (m, 2H), 1.7 (m, 4H), 1.5 (m, 4H), 1.4 (m, 2H), 1.2 (s, 10H), 1.05 (q, 2H), 0.95 (d, 2H), 0.8 (t, 2H), 0.75 (m, 20H)

*C<sub>9</sub>H<sub>19</sub>CO-Lys-Thr-Val-Ile-Ile-Glu-NH<sub>2</sub> 7*

12.0 (br s, 1H), 8.0 (d, 1H), 7.9 (d, 1H), 7.85 (t, 2H), 7.7 (d, 1H), 7.5 (m, 3H), 7.0 (s, 1H), 7.9 (s, 1H), 4.9 (s, 1H), 4.2 (q, 2H), 4.1 (t, 3H), 3.9 (q, 1H), 2.7 (t, 2H), 2.2 (t, 2H), 2.1 (t, 2H), 1.9 (m, 2H), 1.7 (m, 4H), 1.5 (m, 4H), 1.4 (m, 2H), 1.2 (s, 14H), 1.05 (q, 2H), 0.95 (d, 2H), 0.8 (t, 2H), 0.75 (m, 20H)

*C<sub>11</sub>H<sub>23</sub>CO-Lys-Thr-Val-Ile-Ile-Glu-NH<sub>2</sub> 8*

12.0 (br s, 1H), 8.0 (d, 1H), 7.9 (d, 1H), 7.85 (t, 2H), 7.7 (d, 1H), 7.5 (m, 3H), 7.0 (s, 1H), 7.9 (s, 1H), 4.9 (s, 1H), 4.2 (q, 2H), 4.1 (t, 3H), 3.9 (q, 1H), 2.7 (t, 2H), 2.2 (t, 2H), 2.1 (t, 2H), 1.9 (m, 2H), 1.7 (m, 4H), 1.5 (m, 4H), 1.4 (m, 2H), 1.2 (s, 20H), 1.05 (q, 2H), 0.95 (d, 2H), 0.8 (t, 2H), 0.75 (m, 20H)

*C<sub>13</sub>H<sub>27</sub>CO-Lys-Thr-Val-Ile-Ile-Glu-NH<sub>2</sub> 9*

12.0 (br s, 1H), 8.0 (d, 1H), 7.9 (d, 1H), 7.85 (t, 2H), 7.7 (d, 1H), 7.5 (m, 3H), 7.0 (s, 1H), 7.9 (s, 1H), 4.9 (s, 1H), 4.2 (q, 2H), 4.1 (t, 3H), 3.9 (q, 1H), 2.7 (t, 2H), 2.2 (t, 2H), 2.1 (t, 2H), 1.9 (m, 2H), 1.7 (m, 4H), 1.5 (m, 4H), 1.4 (m, 2H), 1.2 (s, 24H), 1.05 (q, 2H), 0.95 (d, 2H), 0.8 (t, 2H), 0.75 (m, 20H)

**Synthesis of C<sub>6</sub>F<sub>13</sub>C<sub>2</sub>H<sub>2</sub>COOH (12) <sup>51</sup>**

In a flask equipped with a cooler and a dropping funnel containing 10 g (21 mmole) 1,1,1,2,2,3,3,4,4,5,5,6,6-tridecafluoro-8-iodooctane dissolved in 15 mL dry diethyl ether, 0.52 g (21 mmole) of magnesium turnings and 35 mL dry diethyl ether were placed. While stirring 1 ml of the iodooctane solution was added at room temperature. After the start of the reaction the remainder of the iodooctane solution was slowly added during an hour. After the addition was completed the solution was stirred for 2 hours, during this time the solution became turbid. The solution was cooled with a waterbath to 5 °C and solid carbondioxide was added. The reaction was stirred for 20 minutes resulting in a precipitate. Subsequently 25 mL of a 25% solution of H<sub>2</sub>SO<sub>4</sub> was added and the reaction mixture was allowed to warm to room temperature. The aqueous phase was separated and the diethyl ether was extracted 3 times using a 10 M NaOH solution. The NaOH layers were combined and heated to 100 °C for 15 minutes. Subsequently the aqueous layers were cooled to 0 °C and 50%

H<sub>2</sub>SO<sub>4</sub> was carefully added to yield a white precipitate. After filtration the solid was washed with cold water, and subsequently dried in vacuum. Finally recrystallization from toluene yielded pure white crystals of 4,4,5,5,6,6,7,7,8,8,9,9,9-tridecafluorononanoic acid (3.3 g, 8.4 mmol, 40%). The product was characterized and found to be identical to literature values.

### Synthesis of C<sub>6</sub>F<sub>13</sub>C<sub>2</sub>H<sub>2</sub>CO- Lys-Thr-Val-Ile-Ile-Glu-NH<sub>2</sub> (10)

The peptide was prepared by standard solid phase peptide protocol. Fluorinated tail **12** (3.0 equivalents) was dissolved in DMF and mixed with 3.6 equivalents of HOBt (1M solution in DMF). To this mixture 3.3 equivalents of DIPCDI (1M in DMF) were added and after mixing the solution was immediately added to the resin. After 18 hours the resin was washed with DMF, dichloromethane, methanol and diethyl ether and finally dried *in vacuo*. The peptides were cleaved from the resin by treatment with trifluoroacetic acid/water (95/5, v/v) for 4 hours. After precipitation in diethyl ether the peptide was lyophilized from acetic acid. Maldi-TOF Mass (C<sub>41</sub>H<sub>63</sub>F<sub>13</sub>N<sub>8</sub>O<sub>10</sub>) calc.: 1074.45, obs.: 1097.17 ([M+Na])<sup>+</sup>, 1119.15 ([M+2Na-H])<sup>+</sup>

### Synthesis of C<sub>7</sub>F<sub>15</sub>CO- Lys-Thr-Val-Ile-Ile-Glu-NH<sub>2</sub> (11)

The peptide was prepared described by standard solid phase peptide protocol. Perdecafluorooctanoylchloride (3.0 equivalents) was dissolved in DMF and DIPEA (6.0 equivalents) was added. This solution was added to the resin and the reaction mixture was agitated for 18 hours. After washing the resin with DMF, dichloromethane, methanol and diethyl ether the resin was dried *in vacuo*. The peptides were cleaved from the resin by treatment with trifluoroacetic acid/water (95/5, v/v) for 4 hours. After precipitation in diethyl ether the peptide was lyophilized from acetic acid. Maldi-TOF Mass (C<sub>40</sub>H<sub>59</sub>F<sub>15</sub>N<sub>8</sub>O<sub>10</sub>) calc.: 1096.41, obs.: 1119.11 ([M+Na])<sup>+</sup>, 1131.10 ([M+2Na-H])<sup>+</sup>

### Fibril formation

Solutions of peptides with a concentration of approximately 1 mg mL<sup>-1</sup> were prepared in a pH 2.5, 20 mM glycine-HCl buffer, in low adhesion Eppendorf tubes. The buffer was filtered through a 0.2 µm filter prior to use. The samples were sonicated at 50 °C for 30 minutes. Not all peptides completely dissolved upon this treatment and therefore all solutions were again filtered through a 0.2 µm GHP membrane filter, after which the concentration was determined as described below. The peptide solutions were incubated for 5 days to allow for fibers to form.

Dilution series were prepared from the filtrated 1 mg mL<sup>-1</sup> solutions of peptide. After dilution the samples were incubated overnight at room temperature.

### Mixed peptide solutions

Clear solutions of fibrils of 1 mg mL<sup>-1</sup> of Ac-KTVIIE-NH<sub>2</sub> and C16-KTVIIE-NH<sub>2</sub> in buffer were mixed in low adhesion eppendorf tubes. The mixture was sonicated at 50 °C for 1 hour, subsequently the sample was slowly cooled to room temperature and stored at room temperature.

Solutions of C<sub>7</sub>F<sub>15</sub>CO-KTVIIE-NH<sub>2</sub> and Ac-KTVIIE-NH<sub>2</sub> were prepared in trifluoroethanol. Solutions were transferred to low adhesion eppendorf tubes to obtain tubes with 1 mg C<sub>7</sub>F<sub>15</sub>CO-KTVIIE-NH<sub>2</sub>, 1mg Ac-KTVIIE-NH<sub>2</sub> and 1 mg of a 1:1 mixture of both. The trifluoroethanol was removed using a nitrogen-flow and subsequent vacuum. The dry peptide was redissolved in 1 mL MilliQ water by sonication at 50 °C for 30 minutes, yielding clear solutions. The solutions were left for 5 days to allow for fibrils to form.

### Determination of resin loading by Fmoc cleavage assay<sup>54</sup>

To determine the loading of Fmoc-protected amino acids on a resin an Fmoc cleavage assay was used. In triplicate 1-2 mg of dry resin (an estimated 1 µmole Fmoc) was weighed. To each sample 3 mL of a piperidine solution (20 % in DMF) was added, the mixture was agitated and left for 20 minutes. Before taking the sample the dispersion was quickly homogenized and the resin was left to settle. UV spectra were recorded of the samples and of the piperidine solution and the absorbance at 290 nm was recorded (Abs). The loading was determined by the following calculation: Fmoc loading (mmole/g) = (Abs<sub>sample</sub>-Abs<sub>ref</sub>)/(1.65 · mg resin).



### Determination of concentration

Acetylated peptide **1** was dissolved in 20 mM glycine-HCl buffer of pH 2.5 to a concentration of 1 mg mL<sup>-1</sup> (0.74 mM) by sonication. From the clear solution a dilution series was prepared. The UV absorption spectrum of the solutions was determined in a 1 mm quartz cell at 220 nm ( $\epsilon/\text{dm}^3 \text{ mol}^{-1} \text{ cm}^{-1}$  4430). The concentration of peptide amphiphile solutions was determined using this known absorption coefficient.

### CD spectroscopy

Samples were measured at 25 °C in a 1 mm quartz cell. Temperature dependent CD measurements were performed in a temperature range from 20 to 90 °C. The temperature was decreased or increased at a speed of 3 °C per minute. The ellipticity was followed at 222 nm at 0.5 nm intervals, while a full spectrum was measured every 10 °C.

### IR spectroscopy

Solutions of fibrils in water were lyophilized and the dry samples were compressed on the ATR crystal with a pressure of 0.5 Kg. Infrared spectra were recorded for 128 scans at 2cm<sup>-1</sup> resolution.

### TEM

A carbon-coated grid was placed on a drop of peptide solution for 5 minutes. The grid was blotted, dried in vacuo and shadowed with platinum under an angle of 45°.

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# 3

## **Disassembling peptide-based fibers by switching the hydrophobic-hydrophilic balance<sup>‡</sup>**

*In this chapter a convenient methodology is described to disassemble peptide fiber assemblies by manipulating the hydrophobic – hydrophilic balance. For this purpose peptides were modified with both a hydrophilic and a cleavable hydrophobic moiety, of which the overall balance of non-covalent interactions was still in favor of peptide assembly. After cleaving off the hydrophobic tail, the peptide fibers spontaneously disassembled.*

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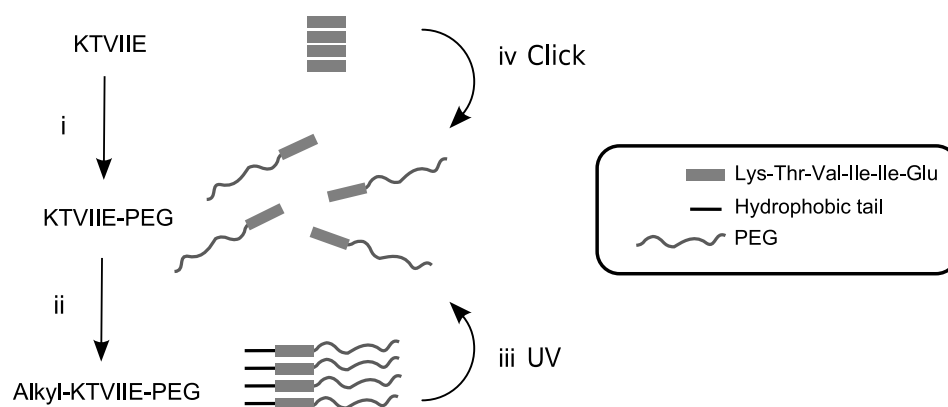
<sup>‡</sup> Part of this chapter has been published: J.T. Meijer, M.J.A.G. Henckens, I.J. Minten, D.W.P.M. Löwik, J.C M. van Hest; *Soft Matter* **2007**, 3, 1135-1137

### 3.1 Introduction

Alkylated peptides represent a versatile class of self-assembling building blocks, due to the subtle interplay between directional hydrogen-bonds and hydrophobic interactions.<sup>1-3</sup> This versatility has recently been explored in a variety of applications. For example Stupp *et al.* have demonstrated the usefulness of peptide amphiphile assemblies for the construction of bioactive materials.<sup>4-7</sup> Furthermore, well defined peptide aggregates have also been used to direct and organize assemblies of synthetic polymers to enhance material properties.<sup>8-10</sup> One of the interesting aspects of peptide assembly is that it can be affected by changing the balance between the non-covalent forces that govern the supramolecular architecture. Induction of peptide assembly has in this respect already been shown using light,<sup>11-13</sup> chemical switches<sup>14,15</sup> and enzymes.<sup>16-18</sup> In all of these cases, changes in hydrophobicity lead to an increased inclination towards aggregation. In the previous chapter it has been shown that introduction of a hydrophobic tail to amyloid-like fibril forming hexapeptide Ac-Lys-Thr-Val-Ile-Ile-Glu-NH<sub>2</sub> (KTVIIE) also increased the stability of the fibrils and rendered the assemblies amenable to manipulation.<sup>19</sup> Upon attachment of the hydrophobic moiety complete absence of fiber disassembly was observed, even at elevated temperatures (up to 90 °C), whereas the unmodified peptide could be disassembled upon heating.<sup>19,20</sup>

Contrary to triggered peptide assembly, the reverse process, *i.e.* controlled peptide fiber disassembly by changing non-covalent interactions *in situ* has not been studied extensively.<sup>21,22</sup> Such a strategy is worth while pursuing since it could lead to new release mechanisms of bioactive peptide fragments and controlled degradation processes for e.g. biomedical applications.<sup>7</sup> In this chapter we describe a convenient methodology to disassemble peptide fiber assemblies by manipulating the hydrophobic – hydrophilic balance.

The strategy to achieve this goal is depicted in figure 1. The fibril forming peptide KTVIIE is first modified with a hydrophilic PEG moiety (i), to prevent assembly of the peptide. Next an additional hydrophobic moiety is introduced (ii), which causes the overall balance of non-covalent interactions to be in favor of peptide assembly. Removing this stabilizing group *in situ* (iii) should now allow us to trigger the disassembly of the fibrils. In order to investigate this possibility peptides are synthesized, which contain both a hydrophilic PEG moiety and an alkyl chain. In this case the alkyl chain is connected to the peptide via the UV cleavable nitrobenzyl moiety.<sup>23-25</sup> Finally, as an alternative approach to disrupt peptide fibrils the *in situ* coupling of a solubilizing PEG tail to a fibril in solution is explored (iv), using the 2+3 cycloaddition reaction between azides and alkynes.<sup>26</sup>

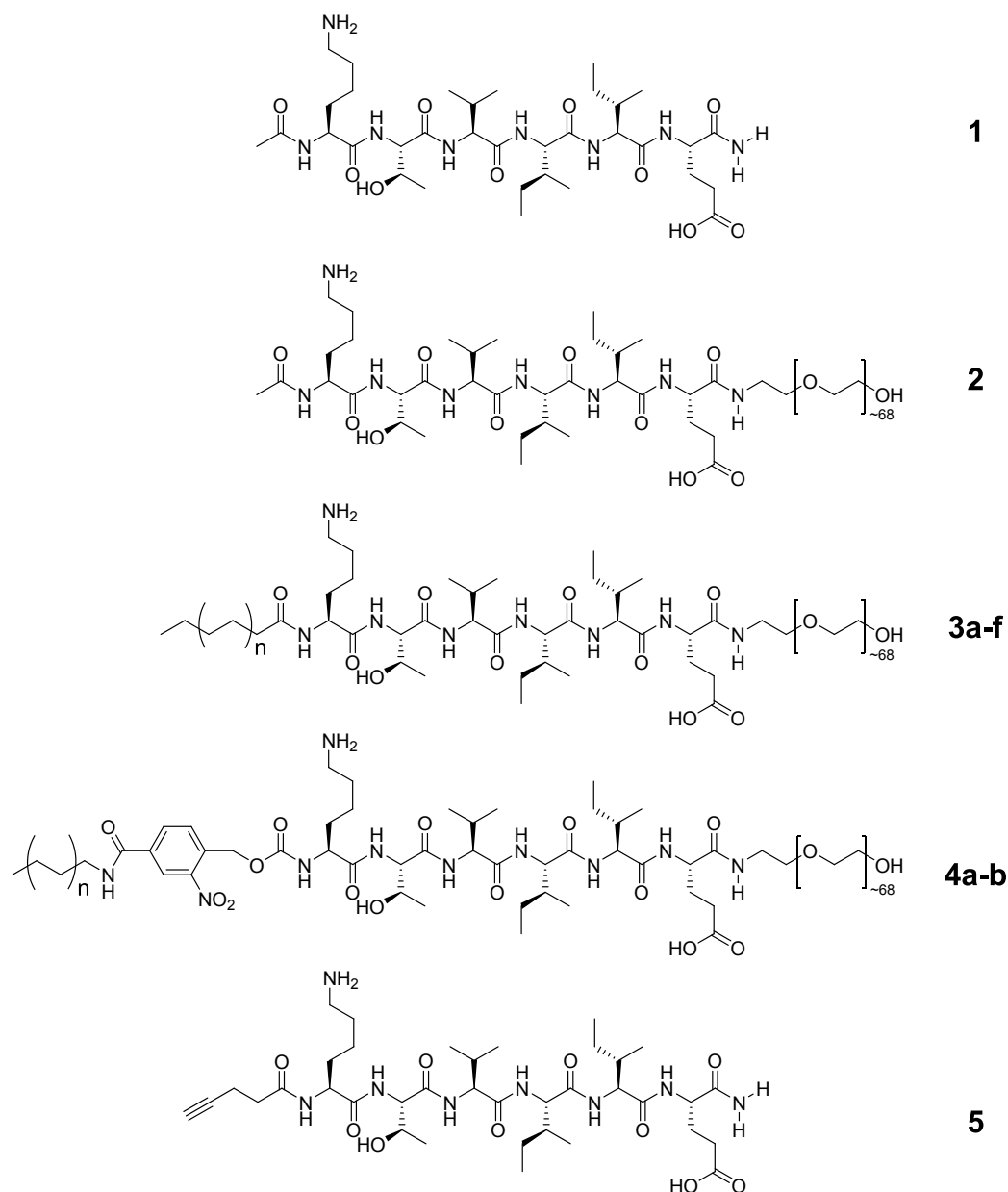


**Figure 1.** Control of peptide fibril stability using hydrophobic and hydrophilic groups; KTVIIE forms amyloid-like fibrils in solution; i) introduction of a PEG tail should prevent assembly ii) introduction of an additional hydrophobic tail should stabilize fibrils; iii) *in situ* removal of the stabilizing tail destroys fibrils; iv) *in situ* introduction of a PEG moiety to KTVIIE destroys fibrils.

## 3.2 Synthesis

### 3.2.1 Synthesis of peptides

In the previous chapter the stabilization of assemblies of the amyloid forming hexapeptide Ac-Lys-Thr-Val-Ile-Ile-Glu-NH<sub>2</sub> was discussed (peptide **1**, Figure 2). It was shown that the introduction of a hydrophobic moiety led to a complete absence of fiber disassembly, even at elevated temperatures (up to 90 °C), whereas the unmodified peptide could be disassembled upon heating.<sup>19,20</sup> Now, we wanted to investigate whether the opposite effect could be achieved by introduction of a hydrophilic group. Therefore, the peptide was synthesized on a resin containing an immobilized PEG moiety of 3 kg mol<sup>-1</sup> by solid phase peptide synthesis following a standard Fmoc-protection group protocol, yielding after cleavage C-terminally modified KVTIIE (figure 2, peptide **2**).



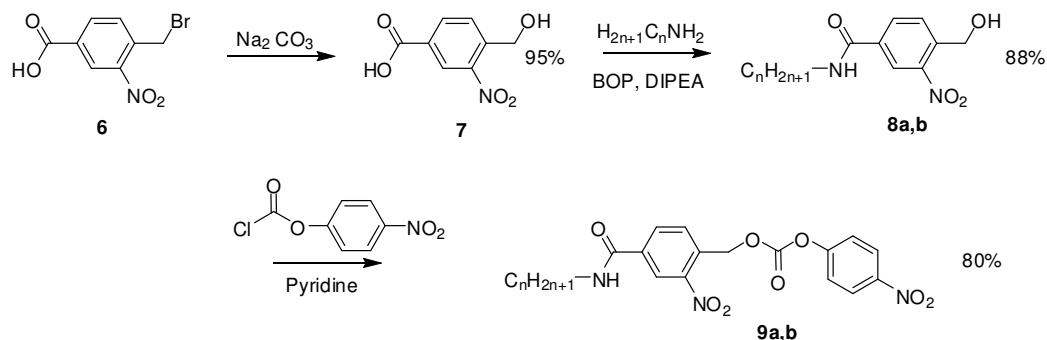
**Figure 2.** Peptides synthesized; Ac-KTVIIE-NH<sub>2</sub> **1**; pegylated hexapeptide Ac-KTVIIE-PEG **2**; amphiphilic peptides: C<sub>2n+4</sub>-KTVIIE-PEG **3a-f** (a: n=1; b: n=2; c: n=3; d: n=4; e: n=5; f: n=6); peptides containing a UV cleavable nitrobenzyl moiety C12-hv-KTVIIE-PEG **4a** (n=5) and C18-hv-KTVIIE-PEG **4b** (n=8); Alkyne-functionalized KTVIIE **5** for in solution functionalization with PEG

In order to establish whether the solubilizing effect of the PEG chain could be counterbalanced hydrophobic tails were introduced N-terminally. The pegylated peptides were synthesized on a resin as described above. After the final amino acid was coupled, an alkyl chain could be introduced efficiently by a classical carbodiimide mediated coupling with the corresponding aliphatic carboxylic acid (peptides **3a-f**, Figure 2). This final coupling was best performed in dichloromethane rather than DMF, due to solubility issues as discussed in

chapter 2. Peptides were cleaved from the resin by treatment with TFA/H<sub>2</sub>O (95/5) for 4 hours. Shorter cleavage times lowered the yield, but more peptide could be removed from the resin repeating the cleavage procedure. The peptides could be precipitated in diethyl ether and lyophilized from acetic acid.

Furthermore, two peptides modified with either a C12 or C18 tail were synthesized containing a UV cleavable linker between the hydrophobic group and the N-terminus (figure 2, structures **4a-b**).<sup>23-25</sup> The UV cleavable hydrophobic tail was synthesized as a single building block in order to facilitate solid phase synthesis (scheme 1). The UV cleavable nitrobenzyl moiety was synthesized following a modified literature procedure.<sup>27</sup> The *p*-nitrophenol carbonate building blocks were synthesized from commercially available 3-nitro-4-bromomethylbenzoic acid, as shown in scheme 1. Also the commercially available 2-nitroterephthalic acid-1-methyl ester was considered as a starting material for the preparation of benzylalcohol **7**. However, procedures to selectively reduce the methyl ester only resulted in products that were difficult to purify and therefore this route was not further explored. Alternatively, benzylbromide **6** was easily transformed into the corresponding alcohol **7** in a 95% yield.<sup>28</sup> Either dodecylamine or octadecylamine were coupled to 4-(hydroxymethyl)-3-nitrobenzoic acid using BOP (benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate) as a coupling agent yielding **8a** and **8b**. The use of BOP prevented any polymerization of **7** as might be expected when employing stronger acylating agents such as N,N'-dicyclohexyl-carbodiimide (DCC). The two different chain lengths were chosen because in advance it was uncertain whether the nitrobenzyl moiety would affect fibril formation. The combination of a C12 chain with the benzyl linker was estimated to be comparable in length with a C16 alkyl chain. The C18 analogue was expected to give more stable assemblies, due to its greater hydrophobicity. C12 and C18 compounds **8a** and **8b** were obtained in yields of at least 86% from carboxylic acid **7**. Finally, *para*-nitrophenol chloroformate was reacted with the alcohol, resulting in the final building blocks **9a** and **9b** after column chromatography in an 80% and 79% yield respectively. The *para*-nitrophenol leaving group allows the tail to react with the free N-terminus of a peptide on the solid phase during synthesis. After the final Fmoc protecting group was removed, **9a** or **9b** was added in dry dichloromethane in the presence of DIPEA. The peptides were cleaved from the resin using TFA/H<sub>2</sub>O, precipitated in diethyl ether, and finally lyophilized from acetic acid.





**Scheme 1.** Synthesis of the UV cleavable hydrophobic tail.

The UV cleavable group was chosen because it met several requirements. First a cleavable site has to be minimally intrusive; it should not inhibit the formation of the fibrils. Furthermore, incorporation between the hydrophobic tail and peptide along the backbone should be chemically compatible with solid phase peptide synthesis and cleavage protocols. Finally, inducing the switch should not affect the rest of the molecule or the assemblies apart from the effect of the removal of the stabilizing moiety.

In a parallel synthesis of the peptides described in Chapter 2 and the pegylated peptides described above the synthesis of the latter proceeded more smoothly; none of the amide couplings and deprotections was troublesome. It is conceivable that the PEG chain might destabilize aggregation of the peptide on the resin, or even prevent interactions between the peptide and the polystyrene based resin.

Finally, in order to be able to couple PEG *in situ* to peptide fibrils via the 2+3 cycloaddition reaction between azides and alkynes (click chemistry)<sup>29,30</sup> pentynoic acid functionalized KTVIIE **5** (figure 5) was synthesized from a Breipohl amide resin, in the same way as the N-alkylated peptides in Chapter 2.<sup>31,32</sup> Pentynoic acid was coupled to the free amine of the peptide on the resin through a classical carbodiimide mediated coupling. Pentynoic acid was used in the functionalization of the peptide because propionic acid was shown to lead to a lot of side reactions in our hands. Furthermore, the slightly longer pentynoyl tail was expected to yield some additional stability to the fibril, as observed earlier for C6-KTVIIE-NH<sub>2</sub> (Chapter 2).

### 3.2.2 Preparation of peptide solutions

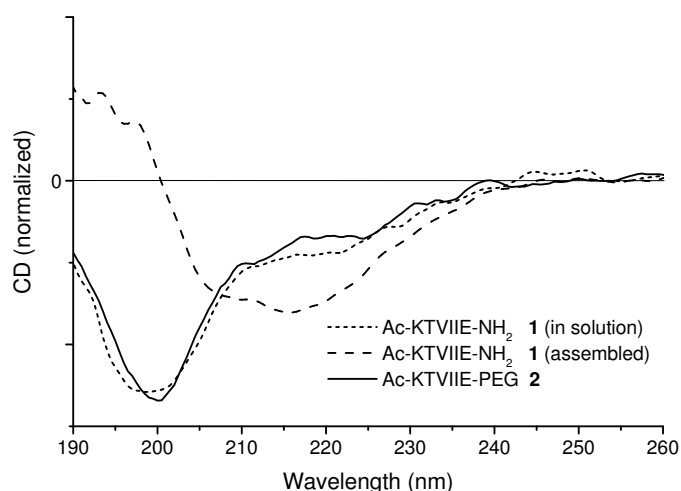
Solutions of the pegylated peptides were prepared according to the following general procedure. Each peptide was dissolved in a 20 mM glycine-HCl buffer (pH 2.5) or MilliQ water to a concentration of 0.6 mM. The samples were sonicated at 50 °C for 30 minutes,

resulting in clear solutions. The peptide solutions were filtered and subsequently incubated for 5 days to allow for fibers to form. CD spectra were identical for solutions prepared in buffer and in water. Because pure water has a larger transmission window in CD this resulted in more informative CD spectra. Furthermore, the absence of salt in the MilliQ solution also improved TEM-sample preparation.

### 3.3 Peptide assembly

#### 3.3.1 Influence of PEG

The presence of fibrillar assemblies in a solution of Ac-KTVIIE-PEG3000 **2** in water was examined using CD spectroscopy and TEM.<sup>33</sup> The peptide showed a CD spectrum with a minimum at 200 nm (Figure 3). The CD spectrum did not change over the course of several weeks. Furthermore, varying the temperature of the peptide solution between 4 °C and 90 °C did not have any effect on the CD spectrum. Increasing the concentration of the peptide from 2 mg mL<sup>-1</sup> to 6 mg<sup>-1</sup> also resulted in similar CD spectra. Furthermore, no fibrils were observed using TEM microscopy.



**Figure 3.** CD spectrum of pegylated peptide **2** showing a random coil type CD spectrum, with spectra of model peptide **1** free in solution and assembled as comparison.

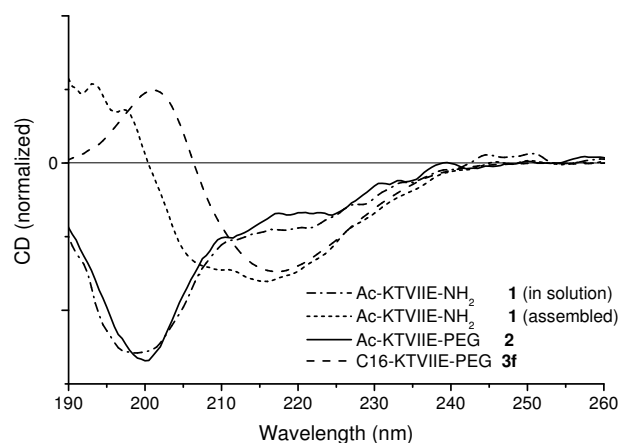
The spectrum of the pegylated KTVIIE peptide was identical to the spectrum of Ac-KTVIIE-NH<sub>2</sub> when it was not assembled in fibrils (chapter 2). This indicated that the peptide was in a random coil fold and therefore in a non-aggregated state. Whereas for peptide **1** a slow self-assembly process was observed,<sup>20</sup> the presence of the PEG chain increased the solubility of the peptide and prevented the assembly into fibrils. The random coil type

spectrum of peptide **2** did not change during several weeks. Ac-KTVIIE-NH<sub>2</sub> showed a minimal assembly concentration of about 0.06 mM, while pegylated peptide **2** did not yet form fibrils at 1.6 mM, which is evidence for a strong inhibitory effect on the self-assembling process.

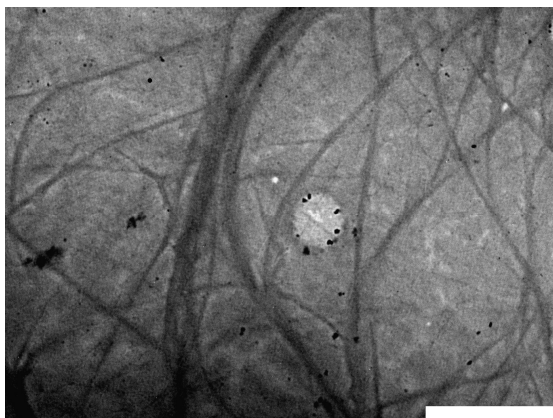
### 3.3.2 Hydrophobic stabilization

Introduction of PEG to the beta-amyloid fibril forming hexapeptide either increased aggregation concentration dramatically or prevented peptide assembly altogether. On the other hand, addition of a C16 tail to KTVIIE lowered the critical assembly concentration from 60  $\mu$ M to below 3  $\mu$ M. Therefore, the introduction of a C16 tail to a KTVIIE-PEG peptide could possibly bring back the ability of the peptide to self-assemble.

The CD spectrum of C16-KTVIIE-PEG (**3f**) showed a CD spectrum with signals at 220 nm and at 200 nm (Figure 4). This CD spectrum is similar to the spectra obtained for the previously examined self-assembling amphiphilic peptides such as peptide **1** above the critical aggregation concentration after they formed fibrils. On heating the solution of **3f** to 90 °C the CD spectrum did not change indicating a stable aggregate of the peptide. TEM confirmed that fibrils were present in the sample (Figure 5).



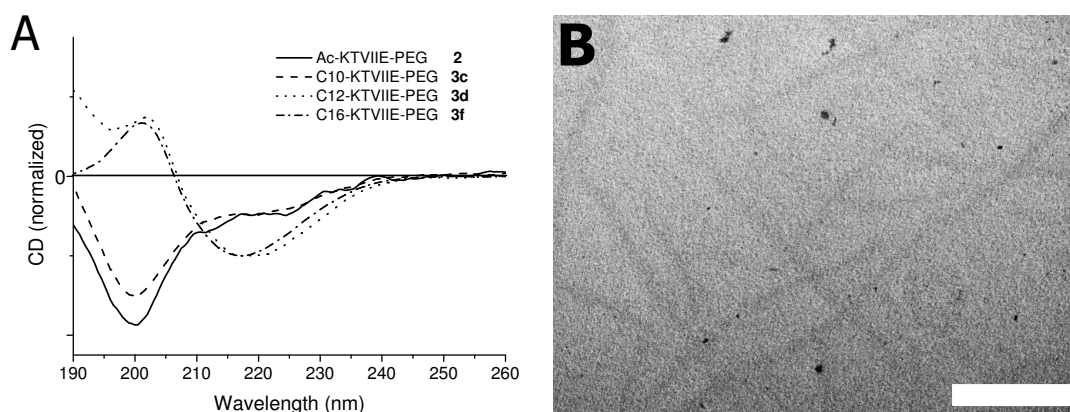
**Figure 4.** CD spectrum of C16-KTVIIE-PEG (**3f**), showing a beta sheet type CD spectrum typical of fibril assemblies, with spectra of peptide **2** and model peptide **1** free in solution and assembled as comparison.



**Figure 5.** TEM picture of fibrils formed by C16-KTVIIE-PEG, bar represents 2  $\mu\text{m}$ .

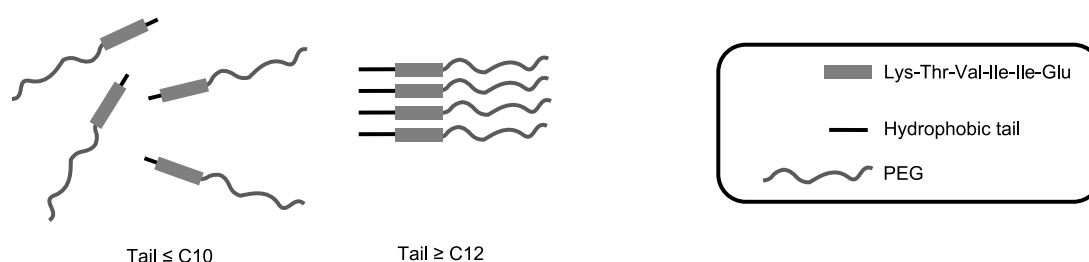
### 3.3.3 Varying the hydrophobic domain

As described above Ac-KTVIIE-PEG **2** does not form assemblies in solution while C16-KTVIIE-PEG **3f** does. In order to establish what hydrophobic domain is required to stabilize fibrils of pegylated KTVIIE, the peptide series **3a-e** was investigated. Introduction of a C6 tail (**3a**) resulted in a CD spectrum with a minimum at 200 nm (Figure 6A), identical to the spectrum of peptide **2**. The same CD spectrum was observed for peptides with a C8 and C10 tail (**3b** and **3c** respectively). Changing the concentration of the solutions or the temperature did not result in changes in the CD spectra. Furthermore no structures were found using TEM microscopy.



**Figure 6.** A) CD spectra of pegylated KTVIIE peptides. Acylated **2** and C10 functionalized **3c** show a spectrum typical for the dissolved species; C12 functionalized **3d** and C16-KTVIIE-PEG **3f** show a beta sheet type CD spectrum typical of fibril assemblies. B) TEM picture of fibrils formed by C12-KTVIIE-PEG (**3d**) bar represents 500 nm.

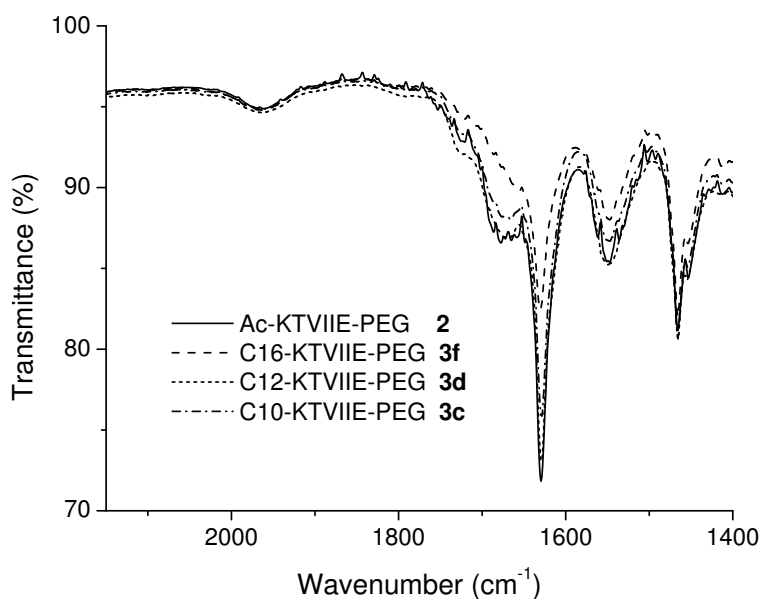
Upon increasing the length of the hydrophobic tail further to C12 (**3d**) the CD spectrum of the peptide had a minimum at 220 nm and a maximum at 200 nm (Figure 6A). This CD spectrum did not change on increasing the temperature to 90 °C. The same was observed for the peptide with a C14 tail (**3e**). In both cases fibrils were present in TEM pictures (Figure 6B). Therefore, the CD spectra showed that the small change in the length of the hydrophobic tail between C10 and C12 resulted in a large change in the assembly properties of the peptides. This suggests that once a critical hydrophobicity is acquired a strong stabilization of the assemblies is established (Figure 7).



**Figure 7.** Influence of a hydrophobic alkyl tail on the N-terminus on the formation of peptide fibrils. Peptides **2** and **3a-c** with tails of C10 and shorter do not form fibrils, while peptides **3d-f** with stabilizing tails longer than C12 do.

To gain more information on the effect of the hydrophobic stabilization on peptide folding infrared spectra were recorded of lyophilized aqueous solutions of peptides **2**, **3c**, **3e** and **3f** (Figure 8). The first two peptides do not form fibrils in solution while the latter two do. Spectra showed bands at  $1630\text{ cm}^{-1}$  and  $1550\text{ cm}^{-1}$ , with a broad signal at  $1675\text{ cm}^{-1}$ . These bands are indicative of a beta-sheet conformation of the peptide (Table I).<sup>34,35</sup> IR spectroscopy on the fibrils in aqueous solution was attempted, but no vibrational bands could be observed.

In contrast to the CD data, the interpretation of the IR spectra was therefore inconclusive, since beta-type amide absorption bands were observed not only in the samples in which fibril-assemblies are present according to CD and TEM, but also in the samples where molecularly dissolved species were expected. Nevertheless, since the KTVIIE sequence has a high tendency to aggregate it is not inconceivable that the peptides will form fibrils at very high concentrations and in the solid state. Therefore, the observed IR spectra on the lyophilized samples do not provide additional information on the assembly behavior in solution that is summarized above in figure 7.



**Figure 8.** Amide region of the IR spectra of Ac (**2**), C10 (**3c**), C12 (**3d**) and C16 (**3f**) KTVIIE-PEG peptides, the first two dissolve while the latter two form fibers in solution according to CD spectroscopy and TEM.

**Table I:** Amide vibrations of peptides **2** and **3c, d, f** ( $\text{cm}^{-1}$ ) with theoretically derived vibration bands for several secondary protein structures.<sup>35</sup>

	Amide I		Amide II	
Random coil	1656 (s)		1535 (s)	
Alpha helix	1650 (s)	1652 (m)	1516 (w)	1546 (s)
Parallel beta	1645 (w)	1630 (s)	1530 (s)	1550 (w)
Anti Parallel beta	1685 (w)	1632 (s)	1530 (s)	
Ac-KTVIIE-PEG <b>2</b>	1675 (w)	1630 (s)	1550 (s)	
C10-KTVIIE-PEG <b>3c</b>	1675 (w)	1630 (s)	1550 (s)	
C12-KTVIIE-PEG <b>3d</b>	1675 (w)	1630 (s)	1550 (s)	
C16-KTVIIE-PEG <b>3f</b>	1675 (vw)	1630 (s)	1550 (s)	

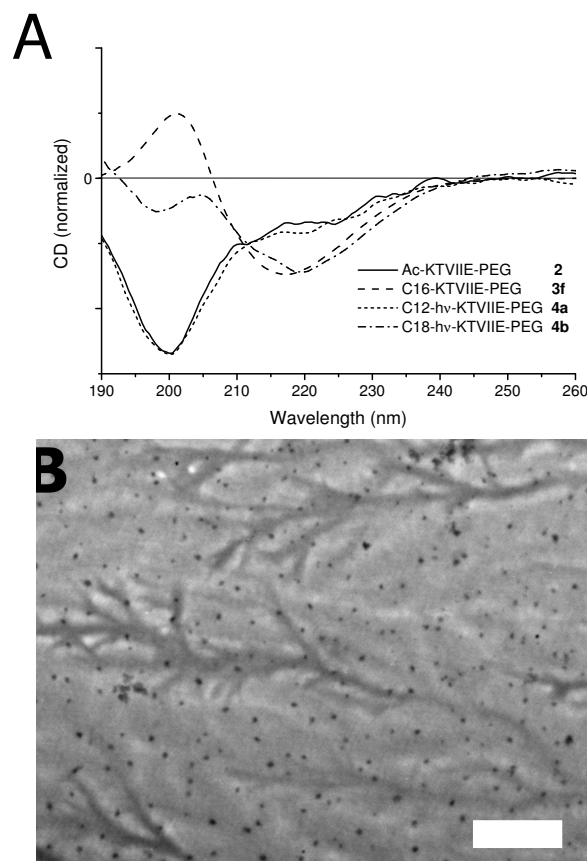
To try and determine the difference in peptide interactions between the fibrils and the dissolved peptides  $^1\text{H}$  NMR spectra were recorded of Ac-KTVIIE-PEG and C16-KTVIIE-PEG in  $\text{D}_2\text{O}$ . In both species the signals of the amino acids were well defined in the spectrum, and the signal of the alkyl tail of C16-KTVIIE-PEG was highly attenuated. This indicates a conformational freedom of the peptide segment of the construct. In contrast, the presence of a strongly packing hydrophobic domain seems to be paramount in the assembly of the amphiphilic species. This is in accordance with the observed minimal C12 length of the hydrophobic domain required to induce fibril formation.

### 3.3.4 Influence of the UV cleavable linker

We postulated the introduction of a UV cleavable group able to remove the fibril stabilizing hydrophobic tail would allow us to disassemble the fibrils. For this purpose a nitrobenzyl based UV sensitive linker was introduced between the hydrophobic tail and the peptide (figure 2). To determine the influence of the introduction of this nitrobenzyl moiety on the formation of fibrils two analogues were prepared, one with a C12 tail (**4a**) and one with a C18 tail (**4b**). The C12 tail should reflect any negative effect of the introduction of the UV cleavable linker as the shortest stabilizing group for the pegylated peptide contained 12 carbon atoms, whereas the C18 tail was expected to introduce a large stabilizing effect on fibrils.

After incubating a solution of peptide **4a** containing the UV-cleavable linker with the C12 tail no beta-sheet type CD signal could be observed; the CD spectrum was similar to the non-fibril forming pegylated peptides (Figure 9). Furthermore, no fibrils could be found using TEM. Increasing the temperature of the solution to 90 °C did not result in a change in the CD spectrum. The influence of the included linker on the assembly probably caused this domain to be too short to stabilize fibrils.

In contrast peptide **4b** with the C18 tail formed the same fibril structures as the amphiphilic peptide **3f**, corroborated by CD spectroscopy and TEM. Apparently, the C18 moiety compensated for any destabilizing effect of the nitrobenzyl linker, as the CD spectrum did not change up to 90 °C. The CD spectrum of cleavable peptide **4b** only differed at 200 nm compared to C16 functionalized peptide **3f** (Figure 9). This is attributed to the presence of the UV cleavable linker, which also absorbs in this region.

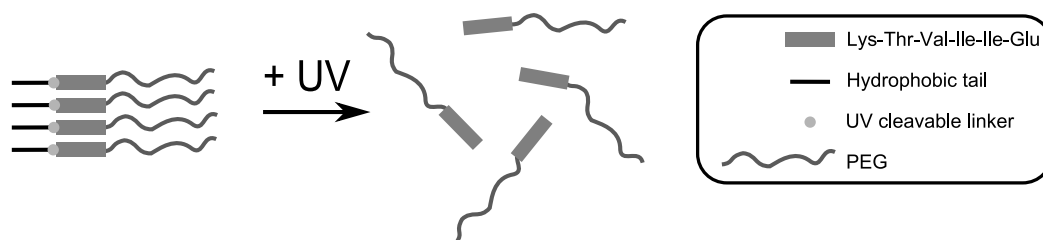


**Figure 9.** A) CD spectra of peptides **2**, **3f**, **4a** and **4b**; B) TEM picture of fibrils formed by C18-hv-KTVIIE-PEG (**4b**) bar represents 2 μm.

### 3.4 Fiber disassembly by UV exposure

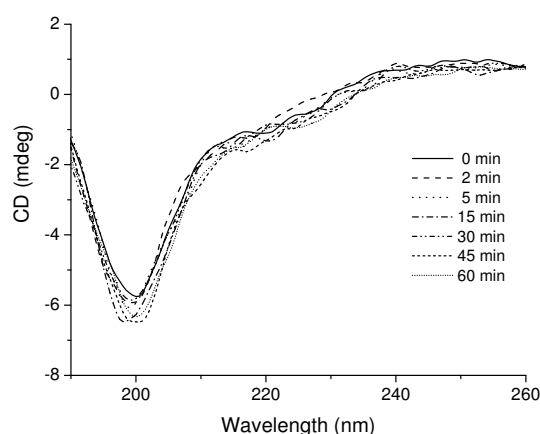
As described above we are able to manipulate the assembly of a fibril forming peptide. Pegylation of hexapeptide KTVIIE prevented aggregation, while subsequent introduction of a hydrophobic n-alkyl group longer than C12 overcame this solubilizing effect. In order to determine the effect and dynamics of the removal of the hydrophobic tail solutions of **4a** and **4b** were exposed to UV radiation and CD spectra were recorded. Typically a peptide solution was placed in a 1 mm quartz cell, 5 cm below the terminus of a quartz lightguide. The shutter was opened for a minute and after exposure the sample was moved to the CD spectrometer to record the CD spectrum. Exposure and CD spectroscopy were repeated for at least an hour or until no further change in the CD spectrum was observed. Exposure times were lengthened up to 15 minutes when the CD spectrum showed no change during the shorter exposures.



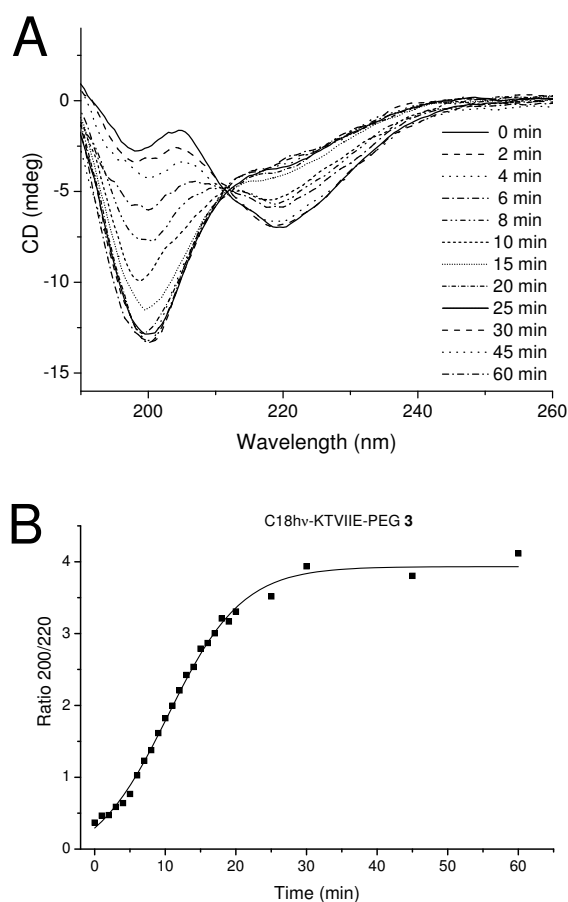


**Figure 10.** Disassembly of Cn-hv-KTVIIE-PEG peptide fibrils by UV initiated removal of a stabilizing hydrophobic tail.

Irradiation of a solution of C12 peptide **4a** with a strong UV-source did not result in any changes to the CD spectrum, even after one hour of irradiation (Figure 11). In contrast, irradiation of assemblies of C18 peptide **4b** resulted in clear changes to the CD spectrum after 3 minutes of exposure (Figure 12a). The ratio of the peaks at 200 and 220 nm increased, characteristic for a change from  $\beta$ -sheet to random coil (Figure 12a). Prolonged exposure changed the CD spectrum completely to a spectrum typical for the dissolved state, showing the destruction of the fibrils which was substantiated by TEM confirming the absence of fibrillar structures. The presence of an isosbestic point in figure 12A indicates a two state mechanism in the change from fibrils to dissolved peptides. During extended waits between cycles of exposure the CD spectrum did not change, showing the dependence of the disassembly process on UV light. Furthermore, sonication of an intermediately disassembled sample did not change the CD spectrum either. The effect of the UV exposure on the cleavable peptide was also monitored via a TLC experiment. The solutions of peptides **4a** and **b** were spotted on the plate before and after exposure to UV. The spots of the complete peptide disappeared during the exposure.



**Figure 11.** CD spectra of C12-hv-KTVIIE-PEG **4a** exposure to UV radiation, the random coil type signal does not change upon exposure up to an hour.

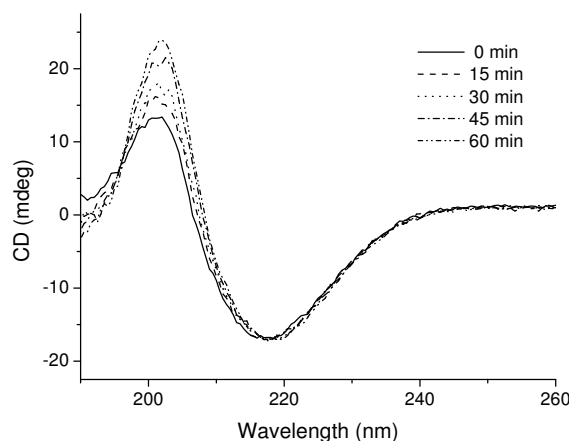


**Figure 12.** A) C18-hv-KTVIIE **4b** upon exposure to UV, showing a transition from a  $\beta$ -sheet like spectrum to a random coil spectrum. B) Peptide C18-hv-KTVIIE-PEG **4b**, ratio of the CD signals at 200 nm and 220 nm upon exposure to UV radiation.

Using the intense UV source complete disassembly of the fibrils took almost an hour of exposure, implying a low reactivity of the linker. In order to ascertain that the observed effect solely depended on this exposure, solutions of peptide **4b** were exposed to different UV

sources. A solution in a 1 mm cell placed for an hour under the UV source used to examine TLC plates (366 nm) did not show a change in the CD spectrum. Furthermore, the CD spectrum of a sample of **4b** that was exposed for 10 hours to 350 nm radiation in a Jasco 810 CD spectrophotometer had not changed.

In a control experiment fibers of peptide **3f** without the labile linker were exposed to the same powerful UV-source for 1 hour. The minimum at 220 nm did not change, but the maximum of the peak at 200 nm increased. This change does not indicate a change in the beta-sheet fold of the peptide in solution, because the signal still represents the beta-sheet fold best. The change in signal could be a result of an increase in temperature of the sample during the exposure to the UV source. The UV exposure therefore did not disrupt the assembly of the fibrils.



**Figure 13.** Exposure of C16-KTVIIE-PEG **3f** to a UV source, showing the persistence of the  $\beta$ -sheet like spectrum.

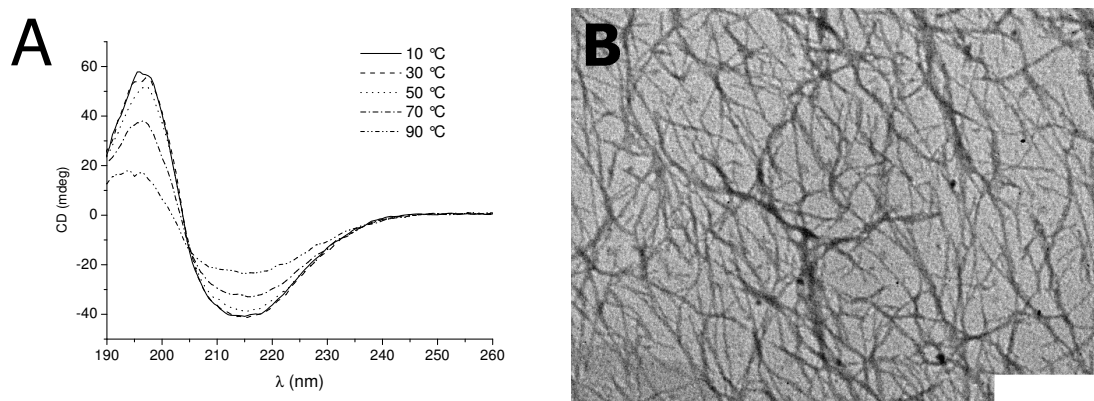
### 3.5 Fiber disassembly by conjugation

As shown above pegylated KTVIIE amphiphiles do not assemble if the hydrophobic tail is shorter than C12. Furthermore, hydrophobically stabilized fibrils can be disassembled by removing the hydrophobic tail. An alternative approach to disrupt peptide fibrils *in situ* would be the coupling of a solubilizing PEG tail to a peptide fibril in solution. The conjugation reaction should be selective and the reaction conditions should be mild enough not to disrupt the fibrils. Moreover, the reaction has to be compatible with the aqueous environment of the fibrils. A reaction that fulfills these requirements is the copper (I) catalyzed alkyne-azide cycloaddition.<sup>36</sup>

The copper catalyzed alkyne-azide cycloaddition resulting in triazoles is a variant of the Huisgen 1,3-dipolar cycloaddition.<sup>29,30</sup> The selective Cu(I)-catalyzed reaction takes place under mild biocompatible conditions and in a regioselective manner, which makes this a robust bioconjugation reaction in aqueous systems, often referred to as a 'click' reaction.<sup>36</sup> The reaction has been used for a wide variety of reactions such as functionalization of polymers and proteins and cyclization of peptides.<sup>26,37-40</sup>

### 3.5.1 Assembly of fibrils

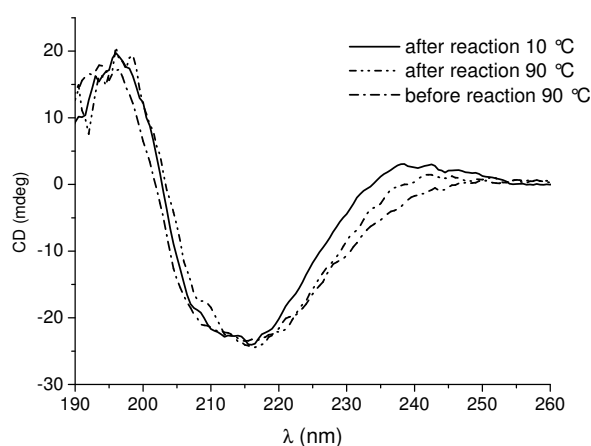
As described above in chapter 3.2.1 alkyne functionalized peptide **5** could be readily synthesized. To ascertain that peptide **5** still formed fibrils in aqueous solution CD spectra were recorded of the solution at room temperature. The CD spectrum showed a negative peak at 220 nm and a positive peak at 200 nm indicating the presence of peptide aggregates of a structure similar to Ac-KTVIIE-NH<sub>2</sub> **1**. Furthermore, TEM pictures showed the presence of fibrils with morphology comparable to that of Ac-KTVIIE-NH<sub>2</sub> **1**. In order to determine whether the presence of the alkyne tail influences the stability of the peptide fibrils CD spectra were recorded at various temperatures. Upon increasing the temperature of the solution of peptide **5** from 20 to 90 °C the intensity of the peaks decreased above 50 °C, but the spectrum remained characteristic for a beta-fold of the peptide (figure 14a). Upon cooling the change in the spectrum was fully reversible. This stabilization of the fibrils by the alkyne tail is similar to the behavior of C8-KTVIIE as described in chapter 2. Since a C12 tail was necessary to form fibrils of pegylated KTVIIE as described in 3.4 above, we still expected the fibrils of peptide **5** to disassemble upon conjugation with PEG.



**Figure 14.** A) Alkyne functionalized KTVIIE **5**, CD spectra at various temperatures, the minimum at 215 nm and the maximum at 195 nm indicate the presence of fibrils at all temperatures; B) TEM picture of fibers formed by peptide **5**, bar represents 500 nm.

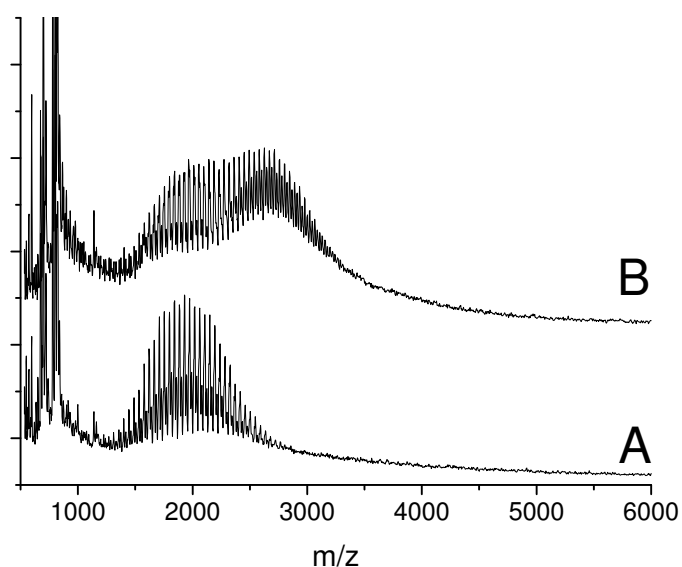
### 3.5.2 Clicking: conjugation of PEG

To a solution of fibrils of peptide **5** in water one equivalent of PEG-azide ( $2.0 \text{ kg mol}^{-1}$ ) was added. To this mixture one equivalent of copper(II)sulfate and 1.5 equivalents of sodium ascorbate were added and the mixture was diluted with water to a final peptide concentration of 0.3 mM. As a control a sample was prepared lacking the copper sulfate. The reaction mixtures were left to stand at room temperature. CD spectra of the reaction mixture containing the copper catalyst were recorded at regular intervals during 18 hours, the CD spectrum of the fibers in the reaction mixture containing copper initially changed. Specifically, immediately after addition of all reagents the CD spectrum resembled that of the peptide **5** at 90 °C. During the 18 hours however, no more changes were observed. The minimum at 220 nm and the maximum at 200 nm remained and no change in the ratio was observed. Upon heating the sample to 90 °C this spectrum did not change either (Figure 15). Even after a week at room temperature the spectrum did not completely convert to a random coil conformation. The reaction mixture without copper showed a similar behavior, therefore the change in the CD spectrum upon addition of the reagents might be the result of the presence of ascorbate.



**Figure 15.** CD spectra of alkyne functionalized KTVIIE **5** after addition of  $\text{CuSO}_4$ , Ascorbic acid and PEG- $\text{N}_3$ . The spectra were recorded at various temperatures.

To establish whether cycloaddition had occurred mass spectra were recorded from the solutions after 24 hours of reaction. Clearly, an additional mass distribution was observed in the reaction mixture containing the copper catalyst, at a mass that was expected for the reaction product (Figure 16). However, not all of the alkyne and azide groups were converted as both unreacted peptide and PEG-azide could still be observed. This could explain why the CD spectrum of the reaction mixture even after 18 hours of reaction time did not change to a random coil conformation. In contrast in the reaction mixture without Cu no peptide-PEG conjugate was observed.



**Figure 16.** Mass spectroscopy images of A) Peptide **5** mixed with PEG- $\text{N}_3$ , B) Crude reaction mixture of peptide **5** with PEG- $\text{N}_3$ ,  $\text{CuSO}_4$  and sodium ascorbate, showing the distribution corresponding to the product.

Using the click approach through the copper (I) catalyzed alkyne-azide cycloaddition it was possible to introduce a PEG tail to preformed fibrils of the KTVIIE peptide. But the introduction did not disrupt the fibrils present in solution, as indicated by CD spectroscopy. In contrast, peptides C6-KTVIIE-PEG **3a** and even C10-KTVIIE-PEG **3c** with similar hydrophobic and hydrophilic groups as the reaction product of the click reaction did not form fibrils in solution. Mass spectroscopy of the reaction mixture containing copper indicated that not all PEG-N<sub>3</sub> and peptide did react. However, it was expected that even a mixture of dissolved peptides and fibrils should result in a different CD spectrum then observed for the reaction mixture as was shown in chapter 2.3.2. Possibly the stability of the fibrils formed by peptide **5** is too great to be disrupted by the introduction of the PEG chain and brush-like structures are formed. In that case steric effects could be partially responsible for the incomplete reaction. The presence of fibrils after the click reaction could however not be corroborated by electron microscopy.

### 3.6 Conclusion

The introduction of the hydrophilic group to amyloid-fiber forming peptide KTVIIIE inhibited the formation of structured assemblies of this peptide. Upon increasing the hydrophobic character of the peptide by introducing an additional N-terminal alkyl chain the well-structured aggregates return. Interestingly the transition from soluble to fibril forming peptide takes place on a small increase of the hydrophobic character; *i.e.* on an increase of the length of the introduced n-alkyl group from 10 to 12 carbon atoms.

This led to the possibility of controlling the presence of peptide fibrils *in situ*. In a first approach a UV cleavable nitrobenzyl moiety was introduced between the pegylated peptide and the hydrophobic tail. The introduction of this group lessened the stabilizing effect of the hydrophobic group, but this negative effect could be overcome by a lengthening of the tail from C12 to C18. Exposure of the fibrils of the peptide containing the cleavable linker to UV led to the disruption of the assemblies, resulting in dissolved peptide. In a second approach a handle was introduced to the peptide to enable the selective introduction of PEG to pre-formed fibrils. Although this approach resulted in the formation of pegylated peptide the fibrils were not disrupted.

### 3.7 Experimental

#### Methods and materials

<sup>1</sup>H-NMR spectra were measured on a Varian Inova 400 MHz NMR spectrometer. MALDI-TOF spectra were measured on a Bruker Biflex II mass spectrometer, 2,5-dihydroxybenzoic acid (DHB) was used as matrix. Electrospray mass spectra were recorded on a Thermo LCQ or JEOL Accu-TOF. UV-Vis measurements were performed on a Varian Cary 50 UV-Vis spectrophotometer. Peptides were synthesized on a Labortec SP4000 or a Labortec SP640 semi-automatic peptide synthesizer. All reagents were obtained from common commercial sources and used as received. Transmission Electron Microscopy was performed on a JEOL 1010 transmission microscope. A carbon-coated grid was floated on a drop of fibril solution for 5 minutes. The grid was blotted and dried in vacuo.

#### 4-(hydroxymethyl)-3-nitrobenzoic acid 7

This compound was prepared according to a modified literature procedure<sup>41</sup>. 0.26 g (1.0 mmol) 3-nitro-4-bromomethylbenzoic acid **6** and 0.53 g (5.0 mmol) Na<sub>2</sub>CO<sub>3</sub> were dissolved in 4 mL H<sub>2</sub>O and 4 mL acetone. The mixture was heated to reflux for 5 hours. Subsequently, the acetone was removed by evaporation. The residual solution was washed with diethyl ether and concentrated HCl was added until a precipitate was formed at pH 2. The resulting suspension was extracted with ethyl acetate while the pH was kept at 2. The combined organic layers were washed with water, dried over MgSO<sub>4</sub>, filtered and concentrated to a brown solid. The product was purified by column chromatography on silica yielding an orange solid (eluent: dichloromethane, methanol, acetic acid (89/10/1, v/v/v)) (yield: 70%). R<sub>f</sub> 0.26.

Analytical data were in accordance with that reported.<sup>41</sup> <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>): δ ), 13.55 (s, 1H, COOH), 8.45 (d, 1H, Ar-H), 8.25 (dd, 1H, Ar-H), 7.95 (d, 1H, Ar-H), 5.67 (s, 1H, CH<sub>2</sub>OH), 4.86 (s, 2H, CH<sub>2</sub>-OH).



**4-(hydroxymethyl)-3-nitro-N-dodecylbenzamide 8a**

0.20 g (1.0 mmol) 4-(hydroxymethyl)-3-nitrobenzoic acid **7** and 0.19 g (1.00 mmol)  $C_{12}H_{25}NH_2$  were dissolved in dichloromethane (~2.5 mL), 0.44 g (1.00 mmol) benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate BOP and 270  $\mu$ L (2.00 mmol) N,N-diisopropylethylamine were added. The reaction mixture was stirred overnight; pH was kept at 8. The reaction mixture was evaporated in vacuo and the product was dissolved in ethylacetate. The mixture was washed three times with 1M HCl and with water, 1M  $NaHCO_3$ , water and finally brine. The ethyl acetate was dried over  $MgSO_4$  filtered and the solvent was evaporated in vacuo. The product was purified by column chromatography (eluent: dichloromethane, methanol, acetic acid (84/5/1, v/v/v)) to afford a 90% yield.  $R_f$  0.37.  $^1H$ -NMR ( $CDCl_3$ ):  $\delta$  0.88 (t, 3H), 1.26 (m, 18H), 1.64 (qu, 2H), 3.48 (q, 2H), 5.05 (s, 2H), 6.26 (t, 1H), 7.88 (d, 1H), 8.06 (d, 1H), 8.44 (s, 1H).

**4-(hydroxymethyl)-3-nitro-N-octadecylbenzamide 8b**

0.20 g (1.0 mmol) 4-(hydroxymethyl)-3-nitrobenzoic acid **7** and 0.27 g (1.0 mmol)  $C_{18}H_{37}NH_2$  were dissolved in dichloromethane (2.5 mL), 0.44 g (1.0 mmol) benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphoniumhexafluorophosphate (BOP) and 345  $\mu$ L (2.0 mmol) N,N-diisopropylethylamine were added and the mixture was stirred overnight while the pH was kept at 8. The reaction mixture was evaporated in vacuo and the product was dissolved in ethylacetate. The mixture was washed three times with 1M HCl and with water, 1M  $NaHCO_3$ , water and finally brine. The ethyl acetate was dried over  $MgSO_4$  filtered and the solvent was evaporated in vacuo. The product was purified by column chromatography (eluent: dichloromethane, methanol, acetic acid (84/5/1, v/v/v)) to afford a 90% yield.  $R_f$  0.29.  $^1H$ -NMR ( $DMSO-d_6$ )  $\delta$  8.70 1H (t), 8.58 1H (d), 8.25 1H (dd), 7.85 1H (d), 5.60 1H (s), 4.80 2H (s), 3.25 2H (q), 1.5 2H (quint), 1.26 4H (m), 1.20 26H (s), 0.80 3H (t). ESI-Ion trap: Calcd. mw. for  $C_{26}H_{45}N_2O_4 = [M+H]^+$ : 449.34, found: 449.36.

**4-(dodecylcarbamoyl)-2-nitrobenzyl 4-nitrophenyl carbonate 9a**

0.37 g (1.00 mmol) 4-(hydroxymethyl)-3-nitro-N-dodecylbenzamide **8a**, 125  $\mu$ L (1.50 mmol) pyridine and 0.10 g (1.05 mmol) 4-nitrophenyl chloroformate were dissolved in distilled THF and stirred at 0 °C for 2 hours under argon atmosphere. The solvent was evaporated in vacuo and the product was redissolved in dichloromethane. The mixture was washed with brine and 1M HCl, dried over  $Na_2SO_4$  and the volatiles were evaporated in vacuo. Column chromatography, in dichloromethane/methanol (97.5/2.5 v/v), afforded the product as a white solid in a 79% yield.  $R_f$  0.54.  $^1H$ -NMR ( $CDCl_3$ ):  $\delta$  8.53 (d, 1H), 8.29 (dt, 2H), 8.16 (dd, 1H), 7.81 (d, 1H), 7.42 (dt, 2H), 6.53 (t, 1H), 5.76 (s, 2H), 3.49 (q, 2H), 1.65 (qu, 2H), 1.33 (m, 18H), 0.88 (t, 3H).  $^{13}C$ -NMR ( $CDCl_3$ )  $\delta$  164.7, 155.4, 152.2, 147.2, 145.8, 136.4, 133.8, 132.8, 129.4, 125.6, 123.8, 121.9, 67.2, 40.8, 32.1, 29.8, 29.7, 29.5, 27.2, 22.9, 14.3. ESI-TOF: Calcd. for  $[C_{27}H_{35}N_3O_8 + Na]^+$  552.23210, found 552.23218.

**4-(octadecylcarbamoyl)-2-nitrobenzyl 4-nitrophenyl carbonate 9b**

0.48 g (1.00 mmol) 4-(hydroxymethyl)-3-nitro-N-octadecylbenzamide **8b**, 125  $\mu$ L (1.50 mmol) pyridine and 0.10 g (1.05 mmol) 4-nitrophenyl chloroformate were dissolved in distilled THF and stirred at 0 °C for 2 hours under argon atmosphere. The solvent was evaporated in vacuo and the product was redissolved in dichloromethane. The mixture was washed with brine and 1M HCl, dried over  $Na_2SO_4$  and the volatiles were evaporated in vacuo. Column chromatography (eluent: dichloromethane/methanol (97.5/2.5 v/v), afforded the product as a white solid in a 79% yield.  $R_f$  0.54.  $^1H$ -NMR ( $DMSO-d_6$ )  $\delta$  8.80 1H (t), 8.58 1H (d), 8.30 2H (d), 8.25 1H (dd), 7.85 1H (d), 7.58 2H (d), 5.69 2H (s), 3.25 2H (q), 1.5 2H (quint), 1.26 4H (m), 1.20 26H (s), 0.80 3H (t).  $^{13}C$ -NMR ( $CDCl_3$ )  $\delta$  164.7, 155.4, 152.2, 147.2, 145.8, 136.4, 132.8, 129.4, 125.6, 123.8, 121.9, 67.2, 40.8, 32.1, 29.9(broad), 28.81, 29.76, 29.6, 29.5, 27.2, 22.9, 14.3. ESI-TOF: Calcd. for  $[C_{33}H_{47}N_3O_8 + Na]^+$  636.326, found 636.322.

**Ac-Lys-Thr-Val-Ile-Ile-Glu-NH<sub>2</sub> 1**

The synthesis of this compound is described in Chapter 2.

**H<sub>2n+1</sub>C<sub>n</sub>CO-Lys-Thr-Val-Ile-Ile-Glu-NH-PEG-OH 2, 3**

The peptides were synthesized from a tentagel PAP resin (Rapp Polymere). The resin was swollen in N,N-dimethylformamide (DMF) for 30 minutes before use. The coupling reactions were carried out in DMF with three equivalents of Fmoc protected amino acid in the presence of N-hydroxybenzotriazole (HOBt; 3.6 equivalents) and diisopropylcarbodiimide (DIPCDI; 3.3 equivalents). The amide coupling was monitored for

completion with a Kaiser Test.<sup>42,43</sup> The Fmoc protecting group was removed by treatment of the resin with a 20% solution of piperidine in DMF (v/v) (3 times 6 minutes). Between each step the resin was washed thoroughly with DMF. The completed peptide was washed with DMF, dichloromethane, isopropanol, dichloromethane and finally diethyl ether and subsequently dried in vacuo. The resin was divided in batches that were further functionalized.

Dry Fmoc-Lys-Thr-Val-Ile-Ile-Glu-NH-PEG-resin was swollen in DMF for 1 hour and subsequently treated with piperidine (20% in DMF v/v, three times 6 minutes). The resin was washed with DMF and dichloromethane. Three equivalents of the appropriate n-alkyl carboxylic acid were dissolved in dichloromethane; HOBt (3.6 equivalents, 1M in DMF) and neat DIPCDI (3.3 equivalents) were added and the resulting mixture was added to the resin. (For the acetyl group 10 equivalents of acetic anhydride and 10 equivalents of pyridine were employed instead) The suspension was agitated overnight. The resin was washed thoroughly with dichloromethane and methanol, finishing with dichloromethane. The final product was cleaved from the resin in 4 hours by treatment with TFA/water (95/5 v/v). The resin was removed by filtration and the peptide was isolated by precipitation in diethyl ether.

A typical <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>) is given for **H<sub>31</sub>C<sub>15</sub>CO-Lys-Thr-Val-Ile-Ile-Glu-NH-PEG-OH 2f**: δ 12.1 1H (br s), 8.15 1H (d), 8.0 3H (m), 7.80 1H (t), 7.65 4H (m), 4.7 1H (s), 4.2 4H (m), 3.9 1H (q), 3.7 2H (t) 3.6 110H (s), 2.8 2H (t), 2.2 2H (q), 2.0 2H (m), 1.9 2H (d), 1.7 4H (m), 1.5 2H (m), 1.4 2H (m), 1.2 28H(s), 1.0 (q, 2H), 1.1 2H (d), 0.75 25H (m).

#### **H<sub>2n+1</sub>C<sub>n</sub>CO-linker-Lys-Thr-Val-Ile-Ile-Glu-NH-PEG-OH 4.**

Fmoc-Lys-Thr-Val-Ile-Ile-Glu-NH-PEG-resin was swollen in DMF for 1 hour and subsequently treated with piperidine (20% in DMF v/v, three times 6 minutes). The resin was washed with DMF and dichloromethane. Two equivalents of nitrophenyl carbonate **9** were dissolved in dry dichloromethane and three equivalents of N,N-diisopropylethylamine were added. The mixture was added to the resin and the reaction mixture was agitated overnight. The resin was washed using dichloromethane, DMF, dichloromethane, methanol and diethyl ether and dried in vacuo. The peptide was cleaved from the resin using a mixture of TFA, water, ethanedithiol and triisopropylsilane (92.5/2.5/2.5/2.5 by volume). The peptide was isolated by precipitation in diethylether. NMR **H<sub>35</sub>C<sub>17</sub>CO-linker-Lys-Thr-Val-Ile-Ile-Glu-NH-PEG-OH** <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>) (δ ppm): 12.1 1H (br s), 8.75 1H (t), 8.50 1H (d) 8.15 1H (d), 8.0 3H (m), 7.80 1H (t), 7.65 4H (m), 7.6 1H (d), 5.4 2H (s), 4.7 1H (s), 4.2 4H (m), 3.9 1H (q), 3.7 2H (t) 3.6 110H (s), 2.8 2H (t), 2.2 2H (q), 2.0 2H (m), 1.9 2H (d), 1.7 4H (m), 1.5 2H (m), 1.4 2H (m), 1.2 30H (s), 1.0 (q, 2H), 1.1 2H (d), 0.75 25H (m)

#### **Alkyne Lys-Thr-Val-Ile-Ile-Glu-NH<sub>2</sub> 5**

The peptide was synthesized using standard Fmoc peptide synthesis on a Breipohl resin.<sup>31,32</sup> Peptide coupling was achieved using 3 equivalents of Fmoc amino acid and diisopropylcarbodiimide (DIPCDI, 3.3 eq.) with N-hydroxy benzotriazole (HOBt, 3.6 eq.) in DMF. The resin was swollen in DMF for 20 minutes prior to use. The Fmoc-group was removed using piperidine in DMF (20% v/v, 3 times 6 minutes). The resin was washed well with DMF and dichloromethane. Three equivalents of the pentynoic acid were dissolved in dichloromethane and 3.6 equivalents of HOBt (1M in DMF) were added. After mixing 3.3 equivalents of neat DIPCDI were added. The solution was added to the resin and the resin was agitated for 18 hours. After washing the resin with DMF, dichloromethane, methanol and diethyl ether the resin was dried in vacuo. The peptide was cleaved from the resin by treatment with trifluoroacetic acid/water (95/5, v/v) for 4 hours. After precipitation in diethyl ether the peptide was lyophilized from acetic acid. The peptides were characterized using <sup>1</sup>H-NMR spectroscopy and Maldi-TOF mass spectrometry. Maldi-TOF Mass (C<sub>37</sub>H<sub>64</sub>N<sub>8</sub>O<sub>10</sub>) calc.: 780.47, obs.: 781.73 ([M+H]<sup>+</sup>) 803.72 ([M+Na]<sup>+</sup>), 819.71 ([M+K]<sup>+</sup>), 825.72 ([M+2Na-H]<sup>+</sup>)

#### **Click to fibrils**

A 1.2 mM solution of peptide **5** was prepared in water and the clear solution was left for 5 days to allow for fibrils to form. Aqueous solutions of N<sub>3</sub>-PEG-OMe (1.8 mM, 2.0 kg mol<sup>-1</sup>), copper(II)sulfate (50 mM) and sodium-ascorbate (130 mM) were freshly prepared. To the solution of peptide **4** N<sub>3</sub>-PEG-OMe (1 eq.), Cu(II) (1 eq.) and ascorbate (1.5 eq) were added and the mixture was diluted with water to obtain a final peptide concentration of 0.30 mM. The solution was mixed well and left at room temperature. Furthermore, a reference solution was prepared without addition of the copper(II)sulfate.

### Fibril formation

Solutions of peptides with a concentration of 1 mg mL<sup>-1</sup> were prepared in Mili-Q water, in low adhesion Eppendorf tubes. The samples were sonicated at 50 °C for 30 minutes and subsequently filtered through a 0.2 µm filter. The peptide solutions were incubated for 5 days to allow for fibers to form.

### CD spectroscopy

CD spectra were recorded performed on a Jasco J-810 spectropolarimeter with Peltier temperature control. Samples were measured at 25 °C in a 1 mm quartz cell. CD measurements were performed in a temperature range from 10 to 90 °C. The temperature was decreased or increased at a speed of 3 °C per minute. The ellipticity was followed at 222 nm at 0.5 nm intervals, while a full spectrum was measured every 10 °C.

### UV exposure

A solution of peptide in a 1 mm quartz cell was placed 5 cm below the UV source, a Bluepoint 2 with a quartz UV guide (dr Hönle, 290-450 nm, 3 mW/cm<sup>2</sup>). The solution was exposed to the source between 1 and 15 minutes, determined by the timer-controlled shutter of the source. The sample was shaken and the CD spectrum was recorded. The procedure was repeated until the total exposure time was 1 hour. Subsequently a sample was taken and transferred to a TLC plate (chloroform, methanol, water 65/25/4, v/v/v), spots were visualized using TDM.<sup>44</sup>

### IR spectroscopy.

IR spectra were recorded on a Thermo Wattson IR300 spectrometer, fitted with a Harrick ATR unit. Solutions of fibrils in water were lyophilized and the dry samples were compressed on the ATR crystal with a pressure of 0.5 Kg. Infrared spectra were recorded for 128 scans at 2cm<sup>-1</sup> resolution.

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# 4

## Manipulation of a non self-assembling peptide<sup>‡</sup>

*The intricately interconnected folding and assembly behavior of an N-terminally acylated peptide, with the sequence Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly, has been tuned by varying its hydrophobic tail and thermal history. The change in interplay between hydrophobic forces and peptide folding allowed the occurrence of different types of aggregation, from soluble peptides with a random coil conformation to aggregated peptides arranged in a  $\beta$ -sheet assembly, which form helically twisted bilayer ribbons. The introduction of a UV-labile linker between the peptide and the hydrophobic tail allowed for controlled disassembly of fibrils.*

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## 4.1 Introduction

Self-assembly is one of nature's mechanisms by which higher order structures are obtained. Two of the main driving forces for self-assembly, hydrophobic interactions and hydrogen bonding, are both present within amphiphilic peptides. The change in interplay between hydrophobic forces and peptide folding allows the occurrence of different modes of assembly. The previous chapters described the introduction of a hydrophobic tail to further stabilize assemblies formed by a self-assembling peptide.<sup>1-3</sup> In contrast, in this chapter the influence of additional hydrophobic interactions on the folding and assembly behaviour of a non-aggregating peptide is examined.

The water-soluble peptide Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly was selected as non-self-assembling peptide. This GANPNAAG sequence is derived from the circumsporozoite (CS) surface protein of the malaria parasite *Plasmodium falciparum*. Specifically, the Asn-Ala-Asn-Pro sequence is repeated 43 times in this protein, and has the propensity to fold in a  $\beta$ -hairpin conformation.<sup>4,5</sup> In the CS-protein this fold is stabilized through the scaffolding provided by the rest of the protein. The free peptide however, is not stabilized in this way and is present in a random coil conformation in solution.<sup>6</sup>

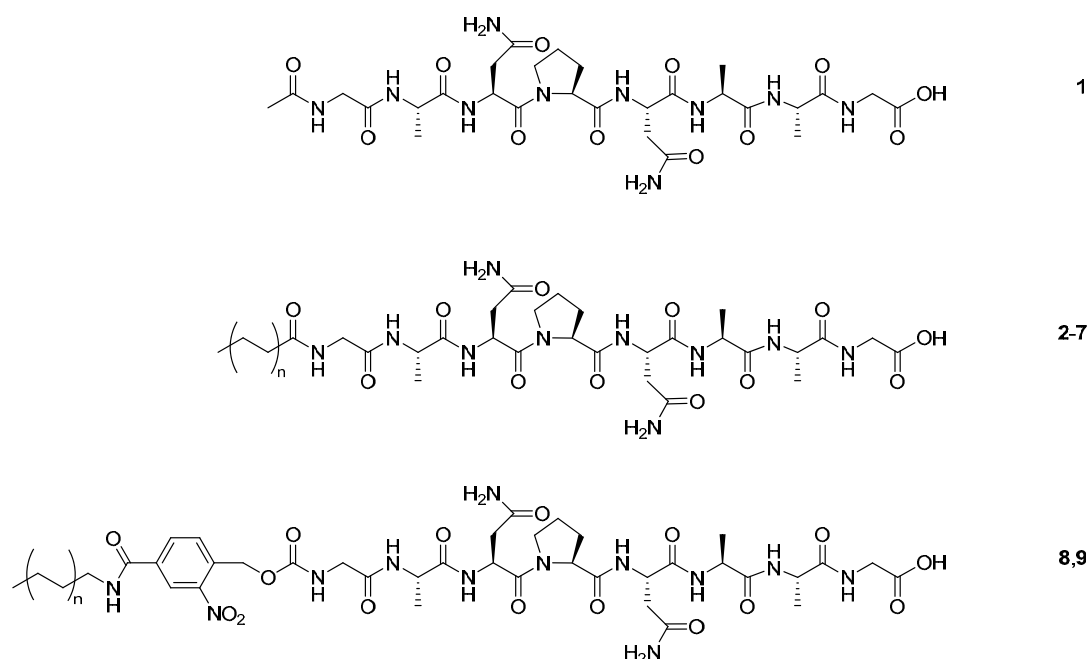
It was shown by Löwik *et al.* that the  $\beta$ -hairpin conformation of the NPNA sequence could be stabilized in a  $\beta$ -hairpin by modifying both N- and C-terminus with alkyl chains and anchoring this amphiphilic peptide in a liposomal scaffold.<sup>6</sup> This stabilizing effect of the double alkylation on the secondary structure of the GANPNAAG peptide indicated it was interesting to examine whether stabilization of the preferred  $\beta$ -turn could also be obtained by introduction of a single alkyl tail. In peptide assemblies, such as peptide amphiphile fibrils, the interactions between the building blocks confer a stable conformation to a peptide. Furthermore, Stupp and co-workers have shown that the recognition of the tightly packed peptide amphiphiles in a peptide fibril is still possible.<sup>7-9</sup>

A series of peptide amphiphiles based on the GANPNAAG peptide was synthesized, the peptides were N-terminally functionalized with a hydrophobic tail. The length of the tail was varied to examine its effect on aggregation behavior and stability of aggregates.<sup>10,11</sup> Furthermore, a UV cleavable linker was introduced in between the hydrophobic tail and the peptide to enable the *in situ* disassembly of peptide amphiphile fibrils.

## 4.2 Synthesis

### 4.2.1 Peptides

The GANPNAAG peptide was conveniently prepared on a Wang resin using a standard Fmoc-based protocol.<sup>12,13</sup> Fmoc protected amino acids were coupled using the carbodiimide DIPCDI coupling agent with HOBt in DMF. The Fmoc group was repeatedly removed using a 20% solution of piperidine in DMF.<sup>14</sup> After the final amino acid was coupled, the alkyl chain could be introduced efficiently by a classical carbodiimide mediated coupling with the corresponding aliphatic carboxylic acid. It was found that the longer alkanoic acids (C16 and C18) employed in the final N-acylation step coupled easier in dichloromethane than in the more polar dimethylformamide, probably because of the higher solubility of the acids and their corresponding activated intermediates. Typically, the *R<sub>f</sub>* values on silica thin layer chromatography plates of the lipidated peptides **2-7** (C6-C18) differed only slightly, whereas reverse phase silica exhibited behavior as expected, with more pronounced differences.

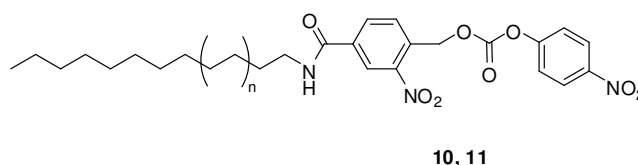


**Figure 1.** Overview of the peptides synthesized, Ac-GANPNAAG **1**; amphiphilic peptides alkyl-GANPNAAG **2-7** (**2**: *n*=2; **3**: *n*=4; **4**: *n*=5; **5**: *n*=6; **6**: *n*=7; **7**: *n*=8); and peptides containing a UV cleavable nitrobenzyl moiety C12-hv-GANPNAAG **8** (*n*=5) and C18-hv-GANPNAAG **9** (*n*=8)



Further, a photo-cleavable linker was introduced through treatment of the GANPNAAG peptide with *p*-nitrophenol carbonates **10** and **11** (figure 2) in the final solid phase step in the preparation of peptide **8** with a C12 tail and **9** with a C18 tail respectively (Figure 1).<sup>15</sup> The two different chain lengths were chosen because in advance it was uncertain whether the nitrobenzyl moiety would affect the fibril formation. The combination of a C12 chain with the benzyl linker (**8**) was estimated to be comparable in length and hydrophobicity with a C16 alkyl chain. The C18 analogue (**9**) was expected to give more stable assemblies, due to its greater hydrophobicity.

These two different chain lengths had previously been used in combination with the  $\beta$ -amyloid fibril forming pegylated KTVIIE hexapeptide as described in chapter 3. For that sequence the introduction of the linker hindered the formation of fibrils for the dodecyl functionalized compound. However, since the GANPNAAG peptide described in this chapter has a different assembly behavior both hydrophobic groups were evaluated again. All peptides were synthesized and fully characterized using NMR and mass spectroscopy.



**Figure 2.** Para-nitrophenol carbonates of the UV cleavable hydrophobic tails, with C12 ( $n = 1$ , **10**) and C18 ( $n=4$ , **11**) alkyl tails.

## 4.2.2 Preparation of peptide solutions

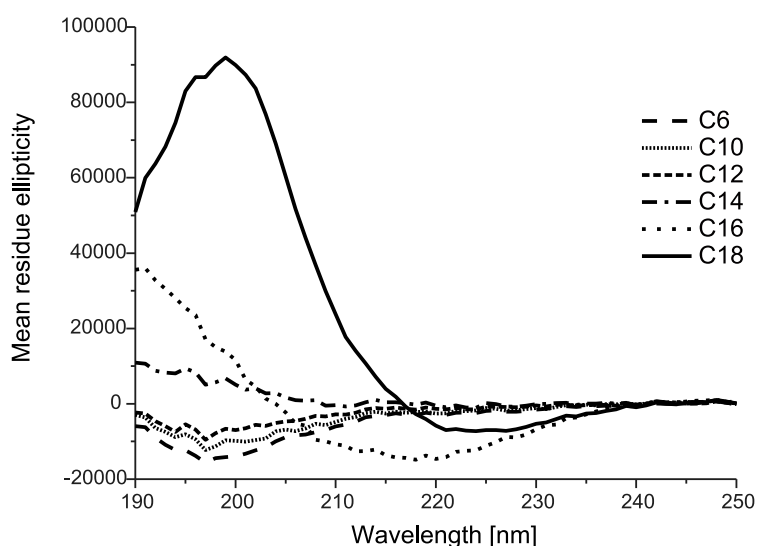
Assemblies of the peptide amphiphiles were obtained by hydration of the samples in MilliQ at 50 °C and subsequent sonication for half an hour at that temperature. This resulted in visually clear solutions for all samples which were stable for several weeks at room temperature, as was evidenced by the reproducibility of their CD spectra. Solutions of C16-GANPNAAG (**6**) formed gels at concentrations higher than 1 mg mL<sup>-1</sup>.

## 4.3 Peptide folding and assembly

### 4.3.1 Influence of an alkyl tail

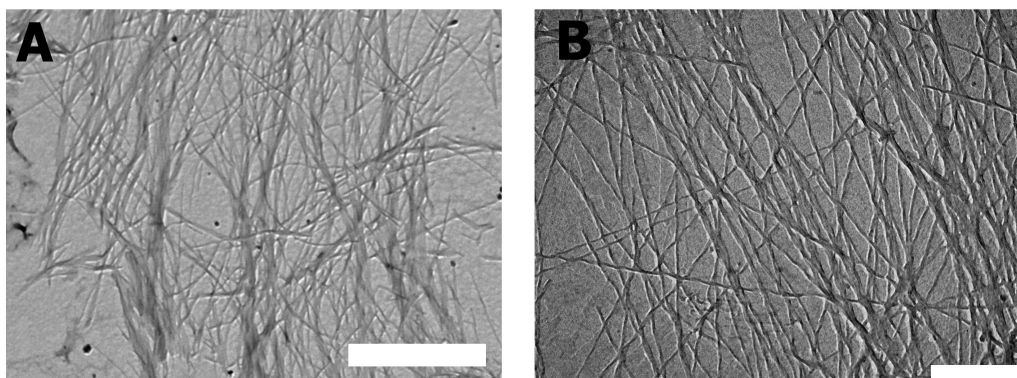
Acetylated peptide **1** showed a CD spectrum with a minimum at 200 nm, typical for a random coil conformation. Also, the CD spectra of C6, C10, and C12 peptides (**2-4**) were indicative of the absence of a structural folding.<sup>16</sup> This could be expected for such short

peptides lacking conformational stabilization via hydrophobic interactions (figure 3). However, increasing the alkyl chain length further it was found by CD spectroscopy that the conformational behavior was affected. At room temperature, the C14 derivative **5** exhibited a  $\beta$ -sheet-type fold, with a minimum at 220 nm and a maximum at 196 nm. Also for the peptides with a length of the hydrophobic tail of 16 or 18 carbon atoms (**6**, **7**) the CD spectra were indicative of a  $\beta$ -sheet fold of the peptide. The high intensity of the signal at 200 nm for peptides **6** and **7** is most probably caused by a linear dichroism component caused by partial alignment of the fibrils due to the container effect of the thin cell used in the CD experiments.<sup>17,18</sup>



**Figure 3.** CD spectra of the GANPNAAG peptide amphiphiles, showing a spectrum typical for a random folding for peptides **1-4** with tails up to C12; a spectrum indicating a  $\beta$ -sheet conformation for C14 peptides **5**; and spectra with  $\beta$ -sheet characteristics for the C16 and C18 functionalized peptides **6** and **7**.

Electron microscopy was utilized to investigate the presence and nature of assemblies formed by the peptides. For the peptides with C12 or shorter alkyl chains, no aggregates could be observed. For C14 fibrous aggregates were found with a 20 nm diameter and micrometers in length but with no other distinctive features. The peptides with C16 and C18 chains formed similar structures, but also twisted ribbons could be observed (figure 4).

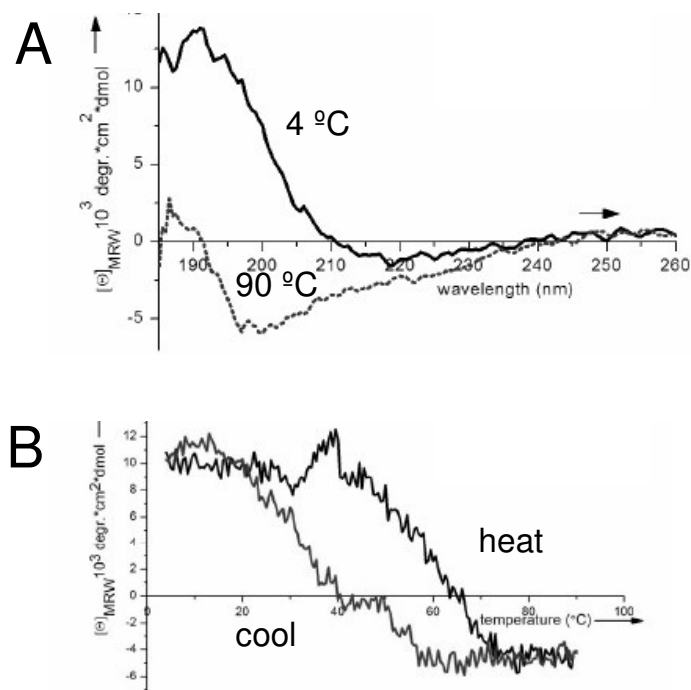


**Figure 4.** TEM pictures of fibrils of a) C14-GANPNAAG **5**; b) C16-GANPNAAG **6**; bars represent 500 nm

The peptide amphiphiles based on the amyloid model peptide KTVIIE discussed in chapter 2 formed fibrils at room temperature independent of the presence of a hydrophobic tail. The morphology of the fibrils formed by KTVIIE did not change on introduction of an alkyl tail at the N-terminus. In contrast, the GANPNAAG peptide did not form assemblies in solution and the introduction of the hydrophobic tail was necessary to induce fibril formation. The fibrillar morphology of the assemblies of **5-7** with tails of C14 and longer was determined by the combination of the hydrophilic peptide and hydrophobic tail. The hydrophobic tails provide the driving force for assembly, while the peptides induce the structure of the fibers.

### 4.3.2 Influence of temperature

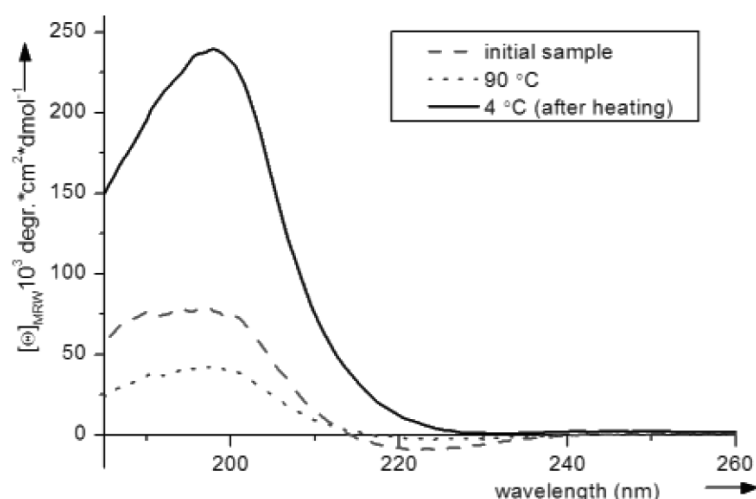
CD measurements at a range of temperatures were deployed to investigate how the stability of the peptide structures was dependent on the nature of the alkyl chains. Upon increasing the temperature from 20 to 90 °C of the solution of peptides with Ac, C6, C10 and C12 tails (**1-4**) the CD spectrum did not change; the peptides remained in a random coil fold.<sup>16</sup> Heating a sample of C14 peptide **5** however, induced a change in the CD spectrum. Between 40 and 70 °C the spectrum changed from a  $\beta$ -sheet to random coil. This change was reversible, upon cooling back to 20 °C the  $\beta$ -sheet spectrum returned (Figure 5).



**Figure 5.** Effect of temperature on the CD spectrum of C14-GANPNAAG (**5**), showing the reversible transition of a signal typical for a  $\beta$ -sheet to random coil. A) CD signal typical for a  $\beta$ -sheet at 4 °C, random coil at 90 °C. B) change in the signal at 200 nm upon first heating the sample and subsequent cooling.

For the C16 peptide **6** the CD spectrum did not change until heated above 80 °C. Strikingly, at 90 °C still some form of organization was maintained for the C16 peptide as was evidenced by the CD signal that had not reverted to that of a random coil but was closer to that of a beta sheet. The state of aggregation at 90 °C was found to have changed, because after cooling the sample exhibited a very strong increase in the CD signal observed around 196 nm, while the optical density of the solution did not change significantly (figure 6).

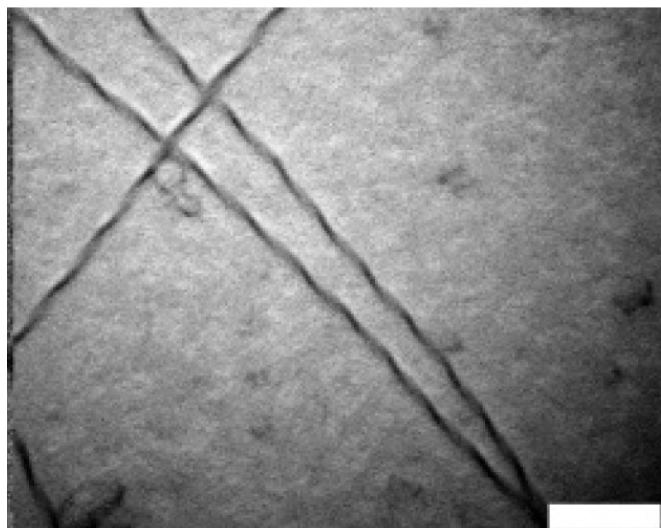
Increasing the alkyl chain further to 18 carbon atoms (peptide **7**) at first afforded structures in which the peptides displayed a CD spectrum similar to that of the C16 compound. However, when these samples were heated above 70 °C, the assemblies already converted into the same structures as those found for the C16 amphiphiles that after heating had been cooled below at least 50 °C. Remarkably, once these samples had undergone a full heating and cooling cycle, further heating almost did not affect the CD spectrum anymore. Only a small decrease in the strong positive effect at 198 nm was observed, reflecting a drastic stabilization of the assemblies that were formed<sup>19</sup>



**Figure 6.** CD spectrum of C16-GANPNAAG **6**, showing an decrease in the intensity of the peak at 200 nm upon heating from room temperature to 90 °C, upon cooling the intensity of the signal increases to a value significantly higher than the starting signal.

A comparison of the CD signals of peptides **6** and **7** with those of helical, sheet, or random coil folded peptides indicated that the Cotton effect could not merely be due to folding of the peptide backbone. The strong increase of the observed signal could be due to, for example, circular intensity differential scattering<sup>20,21</sup> and/or linear dichroism<sup>22,23</sup>. The decrease in intensity of the strong CD signal of peptide **6** with the C16 tail upon heating could be caused by (partial) disassembly or reorganization of the peptide structures.

As described above, upon dissolving the peptides with C16 and C18 chains initially featureless assemblies were formed. However, once the samples of these peptides **6** and **7** had been heated to 90 °C and were cooled again, the assemblies converted into a twisted ribbon configuration, as shown in figure 7, possessing a width of approximately 20 nm. Particularly for the C18 compound **7** the helical fibers were found to be very long (5-10  $\mu\text{m}$ ) and straight, indicative of a high rigidity.



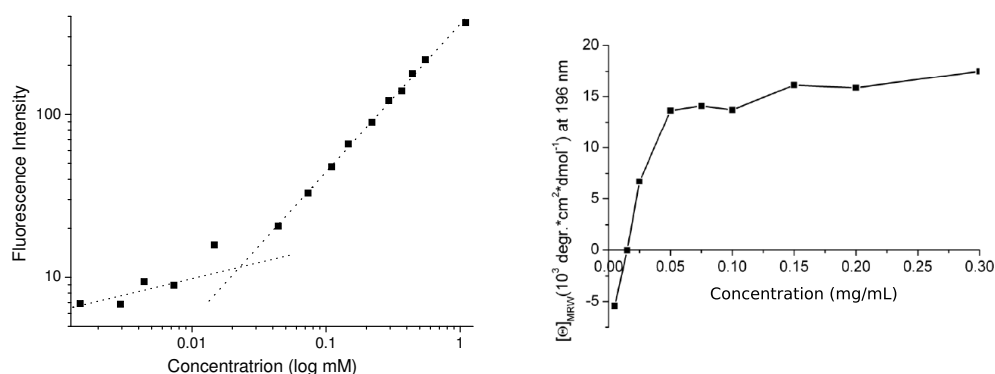
**Figure 7.** TEM picture of C18-GANPNAAG fibrils (**7**) after a heating cycle (bar represents 200 nm)

Similar twisted fibrils have been reported earlier and are assumed to be formed from bilayer sheets that coil into a helical structure,<sup>24,25</sup> however surprising it may be that this type of aggregation seems to be the most thermodynamically stable in an aqueous environment.<sup>26-28</sup> Such assemblies comprise hydrophobic edges exposed to water, which is in agreement with their strong inclination to adhere to carbon-coated grids. The presence of these hydrophobic patches also seems to be reflected in the tendency of further aggregation into bundles of helical fibers, which ultimately form dense networks at higher concentrations.<sup>10,29</sup>

#### 4.3.3 Influence of concentration

In order to determine the critical aggregation concentration of the amphiphilic peptides fluorescence spectroscopy was used following an established procedure. The fluorescence of the probe molecule 1,6-diphenyl-1,3,5-hexatriene (DPH) is low in water and highly enhanced in a hydrophobic environment, for example upon incorporation in a micelle.<sup>19,30</sup> The critical association concentration of C16 peptide **6** was determined using this methodology. A 10 mM stock solution of DPH was prepared in THF, and 1  $\mu$ L of this solution was added to various amounts of peptide **6** in water. The increase in fluorescence above a concentration of  $1.5 \cdot 10^{-5}$  M indicated the presence of aggregates (figure 8). This result was corroborated by CD spectroscopy using a dilution experiment. Below  $3 \cdot 10^{-5}$  M the CD spectrum of C16 peptide **6** resembled that of the water-soluble peptide **1** indicating molecularly dissolved peptides. C18 functionalized peptide **7** did not show a change in CD spectrum at a concentration of  $1.0 \cdot 10^{-5}$

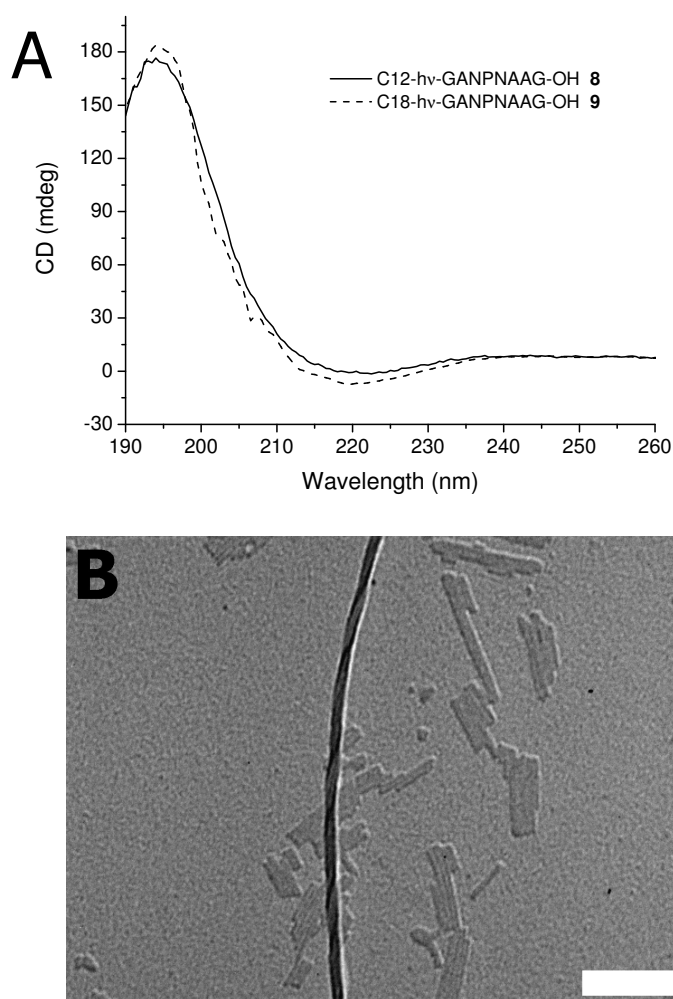
<sup>5</sup>M, below which the CD could not be reliably recorded. The CD signal at 196 nm of the  $\beta$ -sheet type signal was characteristic for the presence of fibrils (figure 8).



**Figure 8.** Determination of critical assembly concentration of C16-GANPNAAG **6** using the fluorescent DPH probe and CD spectroscopy at 196 nm, typical for the  $\beta$ -sheet signal and the presence of fibrils.

#### 4.3.4 The influence of a cleavable linker

As described above a nitrobenzyl moiety was introduced between the GANPNAAG peptide and the hydrophobic tail to allow for controlled disassembly, resulting in peptides **8** and **9** (figure 1). CD spectroscopy was used in order to determine whether peptides **8-9** containing the UV linker formed fibrillar structures comparable to those formed by peptides **6** and **7**. The compounds displayed a very strong positive signal around 196 nm in their CD spectra (figure 9a), which can be related to fibril formation as was shown above. This was confirmed with TEM measurements which revealed the presence of fibrillar structures, as is shown in figure 9b for compound **8**. From these results it can be concluded that the incorporation of the linker had no adverse effect upon fibril formation.

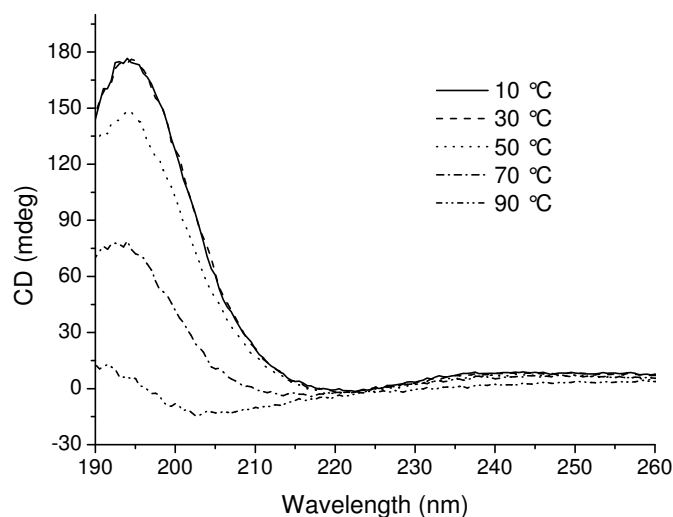


**Figure 9.** A) CD spectra of 1 mM solutions of peptides with C12 (**8**) and C18 (**9**) tails containing the UV cleavable linker B) TEM picture of a fibril of C12-hv-GANPNAAG **8** (bar represents 200 nm)

Typically, the photo-cleavable nitrobenzyl based linker added to the stability of the peptide fibers that were formed, because the same GANPNAAG peptide with only an N-terminal C12 dodecanoyl chain did not form fibrillar structures. Temperature-dependent CD measurements were deployed to investigate whether the stability of the peptide structures was affected by the presence of the UV cleavable linker. On increasing the temperature of a solution of C12 peptide **8** from 20 to 90 °C the intensity of the strong signal at 195 nm decreased (Figure 10), comparable to the behavior of C16 peptide **6** described earlier. Above 70 °C the resulting CD spectrum was typical of a peptide in a  $\beta$ -sheet fold. Therefore above 70 °C the CD spectrum of **8** was comparable to the CD spectra obtained for peptide **5** with 14 carbon atoms in the tail and for peptide **6** at 90 °C (figure 6). This confirmed the previous observation that the nitrobenzyl moiety added to the stabilizing effect of the alkyl tail.



Similarly the effect of temperature on the CD signal of C18 peptide **9** was comparable to C18 peptide **7** described above. The strong CD signal of the fibrils at 196 nm was stable up to 90°C and remained unchanged upon cooling.



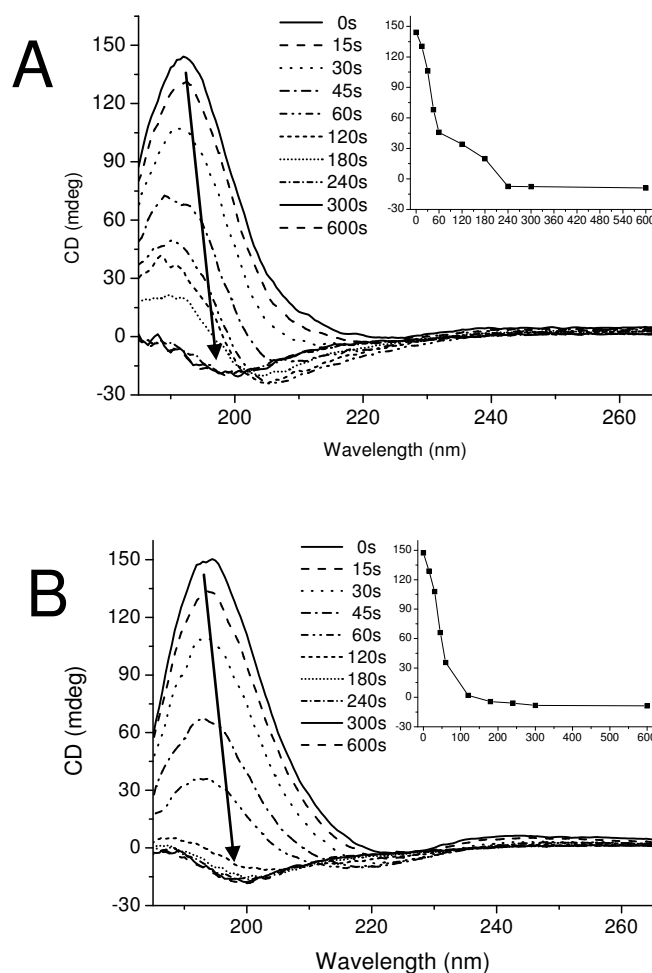
**Figure 10.** CD spectra of C12-hv-GANPNAAG (**8**), upon increasing the temperature from 10 to 90 °C the change in the curve indicates a transition from a  $\beta$ -sheet to a random conformation of the peptide.

Introduction of the nitrobenzyl linker in peptide **8** induced a stabilizing effect to the structures formed by the peptide amphiphile, this is in contrast to the introduction of the same moiety to the pegylated KTVIIE peptide described in chapter 3. The KTVIIE peptide assembled into  $\beta$ -amyloid like structures and the peptide dictated the organization of the fibrils, while the structure of the GANPNAAG amphiphiles is dependent on the interaction between the hydrophobic tail and the soluble peptide. Hence, where for the KTVIIE sequence all amino acids (and interactions) are necessary to form peptide fibrils this is probably not a prerequisite for the GANPNAAG peptide amphiphiles.

#### 4.4 *Fiber disassembly by UV exposure*

The response towards UV-light of fibrils composed of photo-labile peptide amphiphiles **8** and **9** was examined. The fibril solutions were transferred to 1 mm quartz cells and irradiated with a strong UV source for a certain time period, after which a CD spectrum was recorded. In figure 11a it can clearly be seen that for C12 compound **8** the strong CD signal around 195 nm rapidly decreased upon irradiation. After 4 minutes the signal had

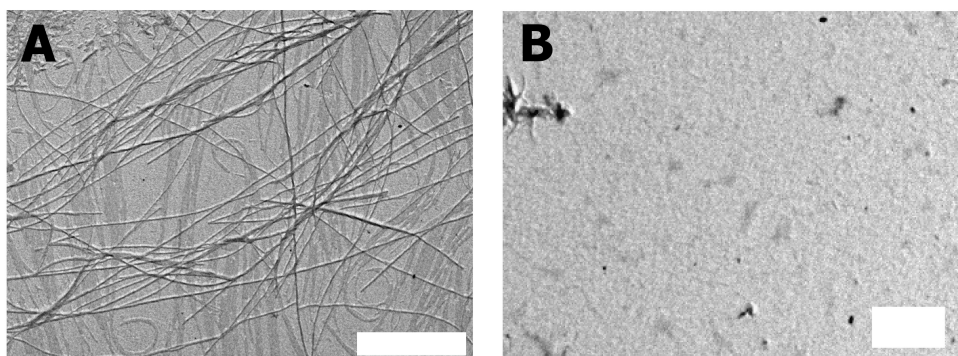
completely transformed into that of a peptide with a random coil conformation, which is expected for the free GANPNAAG peptide in solution.<sup>31</sup> Therefore, it was concluded the fibril structures had been completely disassembled, which was corroborated by the fact that with TEM no structures could be found (fig. 12).



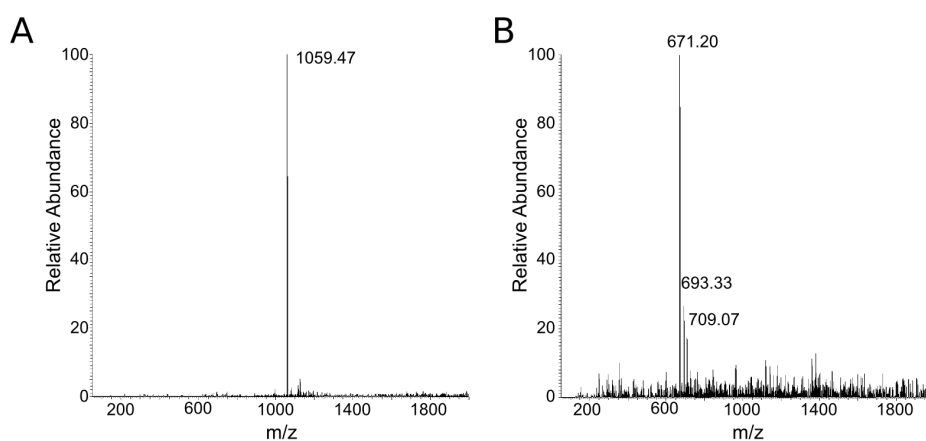
**Figure 11.** CD spectra of C12-hv-GANPAAG (**8**) and C18-hv-GANPAAG (**9**) fiber solutions treated with UV irradiation for different time periods (in seconds, irradiation time increases in the direction of the arrow). Inset: Ellipticity at 192 nm versus irradiation time.

The decomposition of the peptide amphiphile was further evidenced by performing thin-layer chromatography (TLC) of the solutions after different periods of UV irradiation. After 10 minutes all of the starting material had disappeared. The fact that all fibers had disassembled already after 4 minutes, as determined with CD, can be explained by assuming that at that time the peptide amphiphile concentration was reduced below its critical aggregation concentration of 20  $\mu\text{M}$  (*vide infra*), approximately one tenth of the starting concentration.<sup>32</sup> Additionally, the conversion of compound **8** into ‘free’ GANPNAAG **11** was verified by mass spectrometry (figure 13). C18 peptide amphiphile **9** gave results comparable

to peptide **8** upon irradiation with UV. There was no marked difference in the exposure time needed to disassemble the fibrils (figure 11b).



**Figure 12.** TEM images obtained from a C<sub>12</sub>hv-GP (**8**) fiber solution before (A) and after (B) UV irradiation. Bars correspond to 1 and 2  $\mu\text{m}$  respectively.



**Figure 13.** ESI-ion trap mass spectra of a solution of C<sub>12</sub>hv-GANPAAAG (**8**) before (A) and after (B) UV irradiation

Finally, it was established that peptide **6**, lacking the photo-labile linker, was stable under the irradiation conditions described above; no changes were observed in the CD spectra or in the TEM pictures that were taken from the irradiated fiber solutions of **6**. These results indicate that the process of controlled disassembly of peptide amphiphile fibers can be successfully established using a photochemical method, mediated by the photo-labile linker in molecules **8** and **9**.

## 4.5 Conclusion

The introduction of an N-terminal alkyl chain to the soluble GANPNAAG peptide induced the resulting amphiphilic peptide to assemble into fibers with a  $\beta$ -sheet like structure. The stability of these fibrils was altered by modifying the length of the hydrophobic chain, leading to highly stable assemblies for peptides with a C16 and C18 alkyl chain respectively. The introduction of a nitrobenzyl derivative as a labile linker between the peptide part and the hydrophobic alkyl chain of a peptide amphiphile did not affect the formation of structured assemblies. In fact the photo-labile moiety added to the hydrophobic character of the peptide, reducing the length of the N-terminal alkyl chain required for fiber formation from C14 to C12. The removal of the hydrophobic moieties upon exposure of the peptides containing the labile linker resulted in disassembly of the fibrils, opening up the possibility of controlled disassembly of fiber aggregates.

## 4.6 Experimental

### General

All starting materials were obtained from commercial suppliers and used as received. Thin layer chromatography was performed on Kieselgel F-254 pre-coated silica plates or RP-8 F-254s. Visualization was accomplished with TDM<sup>33</sup>. Column chromatography was carried out on Merck silica gel 60 (230-400 mesh ASTM). <sup>1</sup>H-NMR spectra were recorded on a Varian Mercury, 400 MHz. Proton chemical shifts are reported in ppm downfield from tetramethylsilane (TMS). Matrix assisted laser desorption/ionization mass spectra were obtained using DHB (dihydroxybenzoic acid) as the matrix on a Bruker BIFLEX spectrometer. Mass spectra were recorded on a Thermo-Finnigan LCQ Advantage Max (ESI-Ion trap) or a JEOL AccuTOF JMS-T100CS (ESI-TOF). UV/Vis spectra were recorded on a VARIAN Cary 50 Conc spectrometer. CD spectra were recorded on a Jasco J-810 spectropolarimeter equipped with a Jasco PTC-423S/L Peltier type temperature control system. TEM images were obtained with a JEOL- JEM-1010 equipped with a CCD camera. SEM images were created with a Jeol JSM-6330F microscope. Peptides were synthesized on a Labortec SP4000 or a Labortec SP640 semi-automatic peptide synthesizer.

### C<sub>n</sub>H<sub>2n+1</sub>C(O)-Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly-OH 1-7

The GANPNAAG peptide was prepared by standard solid-phase Fmoc protocols. After removal of the final Fmoc protecting group the peptides were capped with either Ac<sub>2</sub>O/DiPEA or the appropriate alkanoic acid dissolved in dichloromethane with diisopropylcarbodiimide as a coupling agent. The product was cleaved from the resin by treatment with TFA/H<sub>2</sub>O (95:5) for two hours followed by precipitation in ether or removal of the volatiles. Column chromatography (eluent: CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O 65:25:4) and subsequent lyophilization afforded pure compounds according to <sup>1</sup>H-NMR, Maldi-TOF and TLC.

Data for C<sub>16</sub>H<sub>31</sub>C(O)-Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly-OH typical for all compounds prepared: Yield: 76%, based on Fmoc-Gly loading (0.7 mmol/g) of initial resin. TLC: R<sub>f</sub> 0.33 (eluent: MeOH/ CHCl<sub>3</sub>/ AcOH 25:65:10). <sup>1</sup>H-NMR [DMSO-d<sub>6</sub>]:  $\delta$  12.45 (broad s, 1H), 8.27 (d, 1H), 7.96 (broad s, 2H), 7.88 (d, 2H),  $\delta$  7.72 (d, 1H), 7.64 (s, 1H) 7.52 (d, 1H), 7.20 (s, 1H), 7.10 (s, 1H),  $\delta$  6.92 (s, 1H), 4.73 (q, 1H), 4.40 (m, 1H), 4.20 (m, 4H), 3.75 (m, 3H), 3.65 (m, 3H), 2.65 (dd, 1H), 2.55 (dd, 1H), 2.40 (m, 2H), 2.18 (m, 3H), 1.85 (m, 3H), 1.48 (m, 2H), 1.20 (m, 30H), 1.12 (d, 3H),  $\delta$  0.85 (t, 3H). Maldi-TOF: Calc. for [C<sub>42</sub>H<sub>72</sub>N<sub>10</sub>O<sub>12</sub> + Na]<sup>+</sup> 931.5, found 931.2.

MALDI-TOF mass spectra peptides 1-9

Compound	Structural formula	(M+H) <sup>+</sup> calc	(M+Na) <sup>+</sup> Obs
<b>1</b>	C <sub>28</sub> H <sub>44</sub> N <sub>10</sub> O <sub>12</sub>	713.31	735.53
<b>2</b>	C <sub>32</sub> H <sub>52</sub> N <sub>10</sub> O <sub>12</sub>	769.38	791.61
<b>3</b>	C <sub>36</sub> H <sub>60</sub> N <sub>10</sub> O <sub>12</sub>	825.44	847.67
<b>4</b>	C <sub>38</sub> H <sub>64</sub> N <sub>10</sub> O <sub>12</sub>	853.47	875.66
<b>5</b>	C <sub>40</sub> H <sub>68</sub> N <sub>10</sub> O <sub>12</sub>	881.50	903.62
<b>6</b>	C <sub>42</sub> H <sub>74</sub> N <sub>10</sub> O <sub>12</sub>	909.58	931.49
<b>7</b>	C <sub>44</sub> H <sub>76</sub> N <sub>10</sub> O <sub>12</sub>	937.56	959.36

### C<sub>12</sub>H<sub>25</sub>C(O)-hv linker-Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly-OH 8

Peptide **8** containing the UV cleavable linker was synthesized following the procedure for peptides **1-7**. After removal of the final Fmoc protecting group the resin was washed with dichloromethane, followed by the addition of two equivalents of 4-(dodecylcarbamoyl)-2-nitrobenzyl 4-nitrophenyl carbonate (**9a** Chapter 3) dissolved in dry dichloromethane and three equivalents of N,N-diisopropylethylamine. The mixture was agitated overnight before the resin was washed subsequently with dichloromethane, DMF, dichloromethane, MeOH and diethyl ether and dried *in vacuo*. The peptide was cleaved from the resin using a mixture of TFA, water, ethanedithiol and triisopropylsilane (92.5/2.5/2.5/2.5 by volume). The peptide was isolated by precipitation in diethylether and purified by column chromatography (eluent: CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O 65:25:4 v/v).

R<sub>f</sub> 0.18 (eluent: CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O 65:25:4 v/v). <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>) δ 8.81 (t, 1H), 8.72 (d, 1H), 8.54 (dd, 1H), 8.21 (dd, 1H), 7.94 (d, 1H), 7.76 (d, 1H), 7.65 (m, 2H), 7.37 (d, 1H), 7.26 (s, 1H), 7.07 (s, 1H), 6.90 (s, 1H), 5.42 (dd, 2H), 4.81 (q, 1H), 4.49 (m, 1H), 4.20 (m, 4H), 3.65 (m, 3H), 3.29 (m, 3H), 2.60 (m, 2H), 2.38 (m, 2H), 2.03 (m, 1H), 1.85 (m, 3H), 1.50 (m, 2H), 1.20 (m, 24H), 1.12 (d, 3H), 0.82 (t, 3H). ESI-TOF: Calcd. for [C<sub>47</sub>H<sub>72</sub>N<sub>12</sub>O<sub>16</sub> + Na]<sup>+</sup> 1083.509, found 1083.506.

### C<sub>18</sub>H<sub>37</sub>C(O)-hv linker-Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly-OH 9

This compound was prepared as described for peptide **8** (using **9b**, from Chapter 3).

R<sub>f</sub> 0.22 (eluent: CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O 65:25:4 v/v). <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>) δ 8.90 (d, 1H), 8.81 (t, 1H), 8.54 (dd, 1H), 8.21 (dd, 1H), 8.18 (d, 1H), 7.95 (d, 1H), 7.76 (d, 1H), 7.63 (t, 1H), 7.60 (d, 1H), 7.29 (d, 1H), 7.25 (s, 1H), 7.05 (s, 1H), 6.89 (s, 1H), 5.42 (dd, 2H), 4.81 (q, 1H), 4.53 (m, 1H), 4.20 (m, 4H), 3.65 (m, 3H), 3.30 (m, 3H), 2.60 (m, 2H), 2.34 (m, 2H), 2.01 (m, 1H), 1.79 (m, 3H), 1.50 (m, 2H), 1.20 (m, 36H), 1.17 (d, 3H), 0.82 (t, 3H). ESI-TOF: Calcd. for [C<sub>53</sub>H<sub>84</sub>N<sub>12</sub>O<sub>16</sub> + Na]<sup>+</sup> 1167.6026, found 1167.6028.

### H-Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly-OH 10

The unmodified peptide **5** was obtained by direct cleavage (TFA/H<sub>2</sub>O, 95:5 v/v, 2 hours) from the resin after removal of the N-terminal Fmoc group. The crude material was used as a reference in mass spectrometry experiments. ESI-ion trap: Calcd. for [C<sub>26</sub>H<sub>42</sub>N<sub>10</sub>O<sub>11</sub> - H]<sup>-</sup> 669.3, found 669.4.

### Sample preparation

Aggregates were obtained by the addition of water to the appropriate peptide amphiphile to obtain a concentration of 1.0 mg/mL. These solutions were left hydrating at 50 °C for 45 minutes followed by 15 minutes of sonication at that temperature. After cooling to room temperature the samples were diluted to their final concentration of 0.20 mg/mL. These samples were found to be stable for at least four weeks as evidenced by the reproducibility of their CD spectra.

In the dilution study the aggregates were sonicated after dilution to their final concentration and their CD spectra measured immediately.

## CD spectrometry

All samples were measured at a concentration of 0.20 mg/mL in a cell with a 1 mm light path. Spectra were recorded with the following settings: speed: 50 nm/min, response time: 2 sec, bandwidth: 1nm, data pitch: 0.5 nm, sensitivity: 1000 mdegrees. Temperature curves were measured at 196 nm at a speed of 1 or 3 °C /min.

## Electron microscopy

Samples for transmission EM were prepared by floating a carbon-coated copper grid on a drop of sample for 5 minutes followed by blotting off the remaining solvent with filter paper and drying in vacuo. The dried grids were platinum shadowed at an angle of approximately 45 degrees. The microscope was set on an accelerating voltage of 60 kV.

## UV irradiation

A solution of peptide fibers in a 1 mm quartz cell was placed 10 cm below the output of a quartz light guide connected to a Bluepoint 2 UV source (Sadechaf UV). The exposure time was determined by the timer-controlled shutter of the source. For the TLC experiment at appropriate intervals a sample was taken and transferred to a TLC plate (eluent: CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O 65:25:4 by volume).

## 4.8 References

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# 5

## **Non-covalent stabilization of a $\beta$ -hairpin peptide into liposomes**

*Alkyl functionalized peptides were bound in the lipid bilayer of a liposome, in order to present stabilized secondary peptide structures, which mimic protein epitopes. As a model system a peptide with the sequence Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly (GANPNAAG) was used, based on a  $\beta$ -turn structure found in the CS protein of the malaria parasite. The peptide was synthesized with alkyl chains attached to both termini, while the length of these tails was varied. The hydrophobic tails stabilized the  $\beta$ -turn of the peptide amphiphiles at the air-water interface of a Langmuir trough. Furthermore, functionalization of the peptide at both termini was shown to not only allow incorporation of the peptide into liposomes, but also to stabilize the  $\beta$ -turn secondary structure in the liposome.*



## 5.1 Introduction

In the previous chapters the influence of hydrophobic tails on self-assembly was discussed, and it was shown that the introduction of additional hydrophobic interactions could stabilize and even induce the formation of peptide assemblies. Besides affecting the aggregation properties of peptides by rendering them amphiphilic, another interesting line of research is to investigate whether also the folding and, hence, secondary structure of peptides can be influenced by the modification with hydrophobic tails.

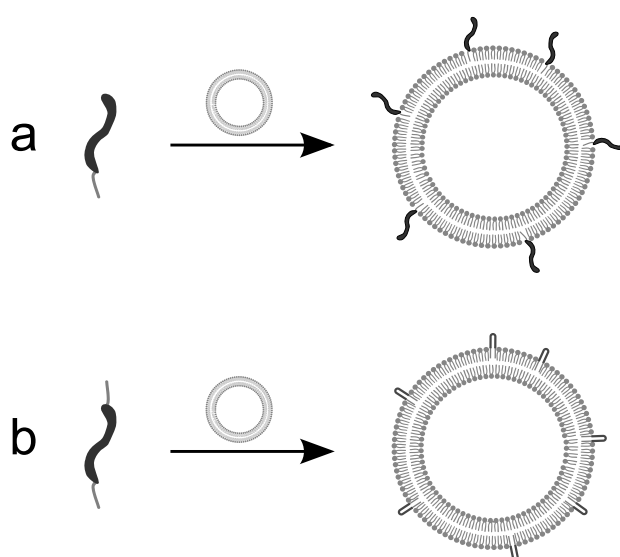
In many biologically relevant interaction processes concerning proteins small parts of the protein, so-called epitopes, are primarily responsible for the recognition event.<sup>1-3</sup> Although the primary sequence is of great importance for the specificity of the recognition, in most cases also the folding of the epitope is crucial for an adequate response.<sup>4-6</sup> When a biologically active peptide is designed to mimic this interaction process, it therefore doesn't suffice to merely use the primary sequence, but also the folding of the peptide has to be obtained.<sup>7,8</sup> In nature the folding is stabilized by the protein itself, which can be considered as a scaffold that keeps the epitope in its right conformation. In biomimetic systems artificial scaffolds have to be designed which allow the introduction of the peptide of interest into its active conformation.

Over the years many different approaches have been developed. Mimics of natural protein binding sites have been prepared by immobilizing linear peptides or stabilized peptide loops on a covalent scaffold.<sup>9-14</sup> The scaffold stabilizes secondary structures and determines the spatial distribution of the peptides.<sup>15</sup> However, these synthetic molecules have been difficult to synthesize.<sup>5,10,11,16</sup> Peptide amphiphiles, comparable in design to the systems described in the previous chapters, have been self-assembled while retaining functionality.<sup>17-21</sup> Furthermore, peptide amphiphiles have been incorporated in liposomes.<sup>22,23</sup> In the bilayer the molecules retain mobility<sup>24</sup> enabling multivalent recognition processes.<sup>25-27</sup> The use of polymerizable lipids allows for the stabilization of self-assembled structures while retaining the functionality of incorporated peptide amphiphiles.<sup>28-30</sup>

In our group Löwik et al. have shown that an epitope can be stabilized in its active conformation by immobilizing an amphiphilic peptide in a dynamic liposomal scaffold, simultaneously presenting the epitope and stabilizing its structure.<sup>31</sup> This was achieved by synthesis of peptides with C18 tails on either the N-terminus or on both the N- and C-termini. Both doubly alkylated and singly alkylated peptides were mixed into DSPC (distearoyl phosphatidylcholine) and this way both peptides were successfully incorporated in liposomes

(figure 1). Using CD spectroscopy it was demonstrated that while the singly alkylated peptide remained in a random coil conformation (figure 1a), the conformation of the doubly alkylated peptide was stabilized into a  $\beta$ -turn (figure 1b).

The epitope that was stabilized was the water-soluble Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly (GANPNAAG) sequence. As described before in chapter 4 this sequence originates from the circumsporozoite (CS) surface protein of the malaria parasite *Plasmodium falciparum*.<sup>32,33</sup> Furthermore, Robinson and coworkers showed that a covalently stabilized loop of this peptide mimics the biologically relevant structure.<sup>34 35</sup>



**Figure 1.** Effect of the incorporation of amphiphilic peptides in DSPC liposomes. a) Incorporated C18-GANPNAAG remains in a random conformation. b) Incorporation of C18-GANPNAAG-C18 results in the stabilization of the peptide in a  $\beta$ -hairpin conformation.<sup>31</sup>

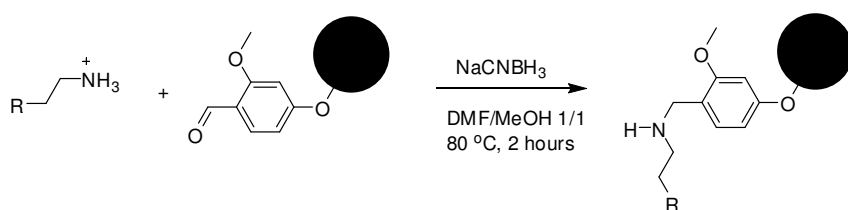
In this chapter the scope of this methodology to stabilize a peptide in a  $\beta$ -turn is examined. Since the stabilization was achieved by the interaction between the peptide alkyl tails and the phospholipid bilayer it was examined how the folding of the GANPNAAG peptide is influenced by the nature of the hydrophobic anchors.<sup>36</sup> For this purpose alkyl tails of different lengths were introduced onto the GANPNAAG peptide. Two approaches were followed for the variation of the hydrophobic moieties. In the first approach the length of the tails at both N- and C-terminus was identical. In the second approach the C-terminus was functionalized with a C18 n-alkyl chain, while the N-terminal alkyl tail was varied in length. Langmuir experiments were performed to determine the influence of the tails on the folding of the peptide amphiphile on the air-water interface. The influence of the interaction between

the hydrophobic tails of the peptide amphiphiles and the bilayer of the liposomal scaffold was examined using phosphatidylcholine lipids with varying hydrophobic tail lengths.

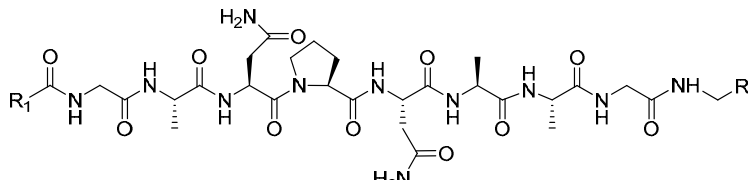
## 5.2 Synthesis

### 5.2.1 Peptide synthesis

In order to introduce alkyl tails at both N- and C-termini of the GANPNAAG peptide (**1-9** table I) a solid phase peptide synthesis protocol was followed. As described in chapter 2, the C-terminal alkyl chain was introduced through reductive amination of a commercially available aldehyde resin with the appropriate alkyl amine (scheme 1).<sup>31,37-39</sup> Subsequently, the first amino acid was introduced using an extended coupling procedure. Finally, the remainder of the peptide, including the N-terminal alkyl tail, was introduced by standard peptide coupling methodologies. The N-terminal alkyl chain was introduced efficiently by a classical carbodiimide mediated coupling with the corresponding aliphatic carboxylic acid. This final coupling was performed in dichloromethane rather than DMF, due to solubility issues as discussed in chapter 2.



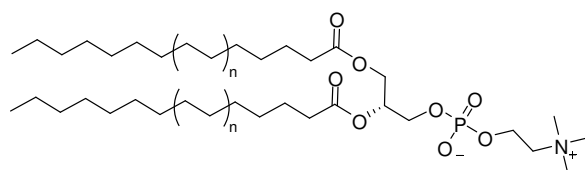
**Scheme 1.** Reductive amination reaction that was used to introduce the C-terminal tail on the resin prior to the synthesis of the peptide. Reaction conditions:  $C_nH_{2n+1}NH_2$ , acetic acid,  $NaCNBH_3$  in  $DMF/MeOH$  (1/1),  $80\text{ }^\circ\text{C}$  for 2 hours. Standard peptide couplings, as described in chapter 2, were used to synthesize the remainder of the peptide sequence.

**Table I:** Overview of the peptides synthesized


Peptide	R <sub>1</sub>	R <sub>2</sub>	Yield (%)
<b>1</b>	C <sub>5</sub> H <sub>11</sub>	C <sub>5</sub> H <sub>11</sub>	63
<b>2</b>	C <sub>11</sub> H <sub>23</sub>	C <sub>11</sub> H <sub>23</sub>	45
<b>3</b>	C <sub>13</sub> H <sub>27</sub>	C <sub>13</sub> H <sub>27</sub>	60
<b>4</b>	C <sub>15</sub> H <sub>31</sub>	C <sub>15</sub> H <sub>31</sub>	82
<b>5</b>	C <sub>17</sub> H <sub>35</sub>	C <sub>17</sub> H <sub>35</sub>	70
<b>6</b>	C <sub>5</sub> H <sub>11</sub>	C <sub>17</sub> H <sub>35</sub>	61
<b>7</b>	C <sub>11</sub> H <sub>23</sub>	C <sub>17</sub> H <sub>35</sub>	60
<b>8</b>	C <sub>13</sub> H <sub>27</sub>	C <sub>17</sub> H <sub>35</sub>	55
<b>9</b>	C <sub>15</sub> H <sub>31</sub>	C <sub>17</sub> H <sub>35</sub>	55

### 5.2.2 Preparing liposomes containing peptide amphiphiles

The conformation of the lipopeptides was studied after incorporation in phospholipid vesicles. In order to determine the effect of the interaction between the different alkyl tail lengths of the peptide amphiphiles and the phospholipid bilayer, phospholipids with varying hydrophobic domains were used. Phospholipids dimyristoyl phosphatidylcholine (DMPC, C14), dipalmitoyl phosphatidylcholine (DPPC, C16) and distearoyl phosphatidylcholine (DSPC, C18) were used in combination with the peptides to form mixed liposomes. These phospholipids are neutral (zwitterionic) molecules with 2 acyl chains of the same length (figure 2).



**Figure 2.** General structure of the phosphatidylcholine lipids that formed the liposomes which were used to incorporate the amphiphilic peptides; DMPC (n=1), DPPC (n=2), DSPC (n=3)

To prepare these functional liposomes both components were dissolved in a mixture of chloroform and methanol (9:1), and subsequently mixed. After removal of the organic solvent the solids were resuspended in water. It was necessary to dry the sample thoroughly in high vacuum in order to remove residual organic solvent before resuspending in water. The

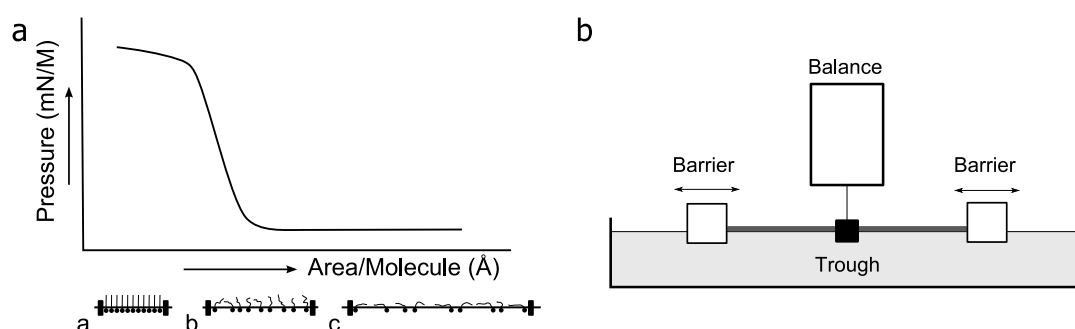
presence of organic solvents during resuspension was found to influence the solubility of the compounds and hence affect the assembly process in an unpredictable way. Typically, solutions were prepared to yield a final concentration of  $1 \text{ mg mL}^{-1}$ , with 10% peptide and 90% phospholipid by weight. These liposome dispersions were stable for several weeks.

Furthermore, the solubility of the peptides in water was examined by determination to what extent dissolved molecules could be detected using CD spectroscopy. To 1 mg of peptide 1 mL of water was added and these samples were sonicated for an hour at  $60^\circ\text{C}$ . Only lipopeptide **1** with two C6 tails afforded a clear solution. The other lipopeptides (**2-9**) remained present as a solid and, after filtration through a  $0.2 \mu\text{m}$  filter, no CD spectrum could be observed.

## 5.3 Peptide folding

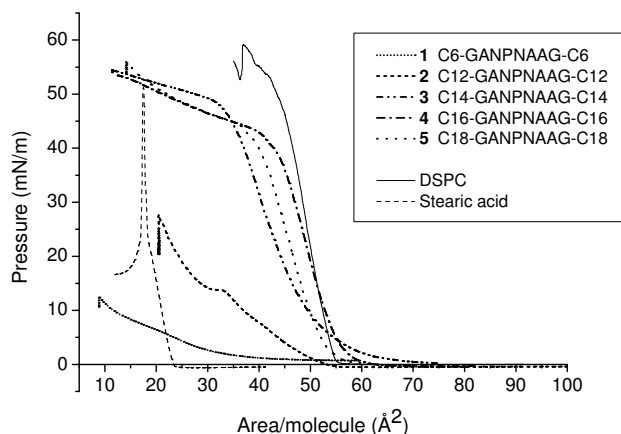
### 5.3.1 Langmuir trough experiments

In order to obtain information on the molecular packing parameters of the peptide amphiphiles Langmuir isotherms were recorded of the lipopeptides on a water-air interface (figure 3). The peptides were dissolved in a chloroform/methanol mixture, and spread on a water surface in a monolayer through. After stabilization and dispersal of the applied molecules the barriers were moved towards each other. The isotherms of stearic acid and DSPC were also recorded as typical representatives of neutral molecules with a minimal area determined by the presence of one or two alkyl tails, respectively.



**Figure 3.** Schematic overview of a Langmuir isotherm measurement. A) Typical compression curve, with the recorded pressure increasing when the area per molecule is lowered due to increased interactions between the amphiphiles. B) The experiment is performed by applying the molecule of interest on the water surface in between two movable barriers; the pressure is recorded through the Wilhelmy plate.

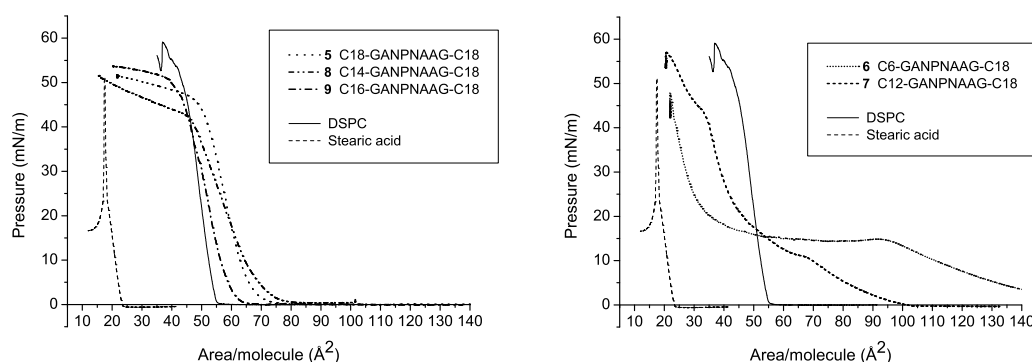
Not unexpectedly, peptide **1** with two C6 tails did not form a stable monolayer due to its solubility in water. Peptide **2** with two C12 aliphatic tails did not form a stable monolayer either; upon stabilizing the barriers at a selected area the registered pressure decreased. Furthermore, the compression isotherm of the C12 compound was not reversible. The other symmetric lipopeptides that were studied (**3-5**), with C14, C16 and C18 alkyl tails respectively, formed stable monolayers on the water surface. They could be compressed into monolayers at the air-water interface of which the area occupied per lipopeptide molecule was determined to be twice the surface of a single n-alkyl carboxylic acid, and comparable to the specific surface of the phospholipid distearylphosphatidylcholine (DSPC). The isotherm of the C18 peptide **5** was reversible as long as the monolayer was not compressed below an area per molecule of  $60 \text{ \AA}^2$ . This corresponds to the area expected for two aliphatic chains pressed together. Once the area per molecule was lowered below this value the monolayer lost its stability and the isotherm became irreversible, which was reflected by a slow decrease of the pressure, showing a collapse of the monolayer. This behavior was also observed for peptides **3** and **4** with C14 and C16 tails respectively.



**Figure 4.** Langmuir isotherms of symmetrical lipopeptides **1-5**; isotherms of DSPC and stearic acid are included for reference. Peptides **1** and **2** with C6 and C12 tails respectively do not form stable monolayers. Peptides **3-5** with the longer tails form stable monolayers at an area per molecule comparable to the phospholipid DSPC.

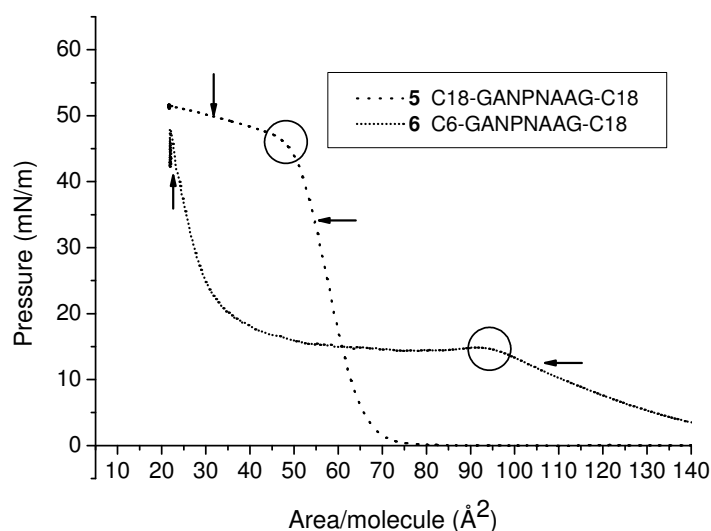
The asymmetric lipopeptides (figure 5a) **8-9** with a C18 tail at the C-terminus and 14 or 16 carbon atoms at the N-terminus, respectively, also formed stable monolayers. The mismatch between the aliphatic chains on the N- and C-terminus did not seem to disturb the stability of the packing. The isotherms of peptides **6-7** with a C6 or a C12 tail revealed a

different behavior. These peptides showed an increase in pressure and, therefore, an interaction at a surface per molecule higher than for **8** and **9**, at  $100 \text{ \AA}^2$  for **7** and even  $140 \text{ \AA}^2$  for **6** in contrast to  $70\text{--}80 \text{ \AA}^2$  for **8** and **9**. Furthermore, the peptides could be readily compressed to an area per molecule smaller than  $55 \text{ \AA}^2$  which is the calculated value for the peptide folded in a tight  $\beta$ -hairpin structure. Isotherms showed that monolayers of **6** and **7** could be reversibly compressed down to an area per molecule approaching the area occupied by one aliphatic tail.



**Figure 5.** Langmuir isotherms of asymmetrical lipopeptides **6-9**; isotherms of DSPC and stearic acid are included for reference. A) Peptides **8** and **9** with C14 and C16 tails respectively show a stable monolayer at an area comparable to DSPC and symmetrical peptide **5**. B) Peptides **6** and **7** with C6 and C12 tails respectively show an interaction at a higher area per molecule, furthermore these peptides could be compressed to an area that was lower than expected for two alkyl tails.

Monolayers of the amphiphilic peptides were transferred to a quartz plate through a Langmuir-Schaefer transfer. CD spectroscopy was employed on these transferred monolayers to gain further information on the conformation of the peptide in the compressed monolayers. The formation of a closely packed monolayer was indicated by a bend in the isotherm, transfers were made at an area per molecule higher and lower than this transition. Recorded CD spectra of peptides with C14 tails or longer, both symmetrical and asymmetrical (**3**, **4**, **5**, **8**, **9**), showed a maximum at 200 nm typical of a  $\beta$ -sheet conformation. These peptides all formed a stable monolayer at a surface per molecule of  $60 \text{ \AA}^2$  and the CD spectrum of the transferred monolayer was independent on whether the transfer was made before or after this transition.



**Figure 6.** Schaefer transfers of the Langmuir monolayers were performed at the compression ratios indicated by the arrows. For peptides that formed a stable monolayer at an area of  $60 \text{ Å}^2$  and peptide **2** with two C12 tails monolayers were transferred just below and above this critical area (circle). For peptides **6** and **7** isotherms showed a first transition above  $60 \text{ Å}^2$  (circle); transfers were made above this transition and at an area per molecule of  $20 \text{ Å}^2$ .

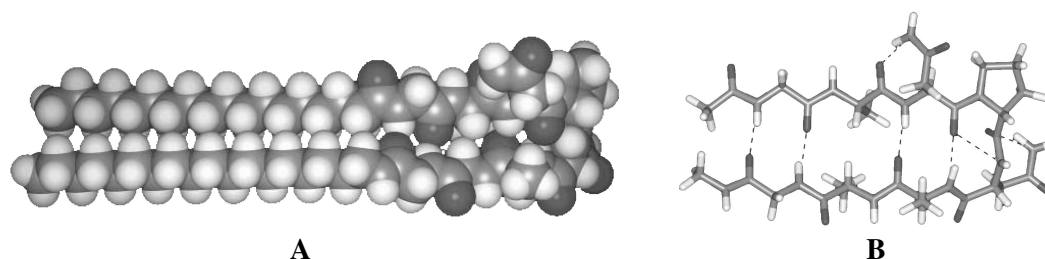
The Langmuir isotherm of C12-GANPNAAG-C12 (**2**) showed a transition at  $33 \text{ Å}^2$ . A transfer of the monolayer at  $35 \text{ Å}^2$  showed a CD signal similar to the amphiphilic peptides with the longer tails, with a maximum at 200 nm and a minimum at 217 nm. In contrast, a transfer of a monolayer compressed to  $20 \text{ Å}^2$  per molecule showed a spectrum with a high signal at 200 nm, but lacking the minimum at 215 nm that is indicative of a  $\beta$ -sheet spectrum. A similar CD spectrum was observed for the monolayers of asymmetrical peptides **6** and **7**, with C6 and C12 chains on the N-terminus respectively, after a transfer at a compression to  $20 \text{ Å}^2$ . For neither peptide, transfers at an area per molecule higher than this resulted in a CD spectrum.

In order to get further insight into the contribution of the peptide to the specific area per molecule at the compressed monolayer Langmuir monolayer experiments were carried out with the mono-alkylated peptides described in chapter 4. However, with none of these peptide amphiphiles reproducible isotherms could be obtained, as at any area per molecule ratio the pressure readily decreased in time. Therefore, it was concluded that these monoalkylated peptide amphiphiles were not able to form stable monolayers. This is probably due to the solubility of the monoalkyl peptides with tails of C12 or shorter and the formation of soluble aggregates by the C14, C16 and C18 peptides. Nevertheless, as a general trend an increase in



the length of the hydrophobic tail corresponded to a higher obtainable pressure. This is probably a reflection of the higher energy barrier for these longer alkyl chain peptides to become dispersed in the aqueous phase.

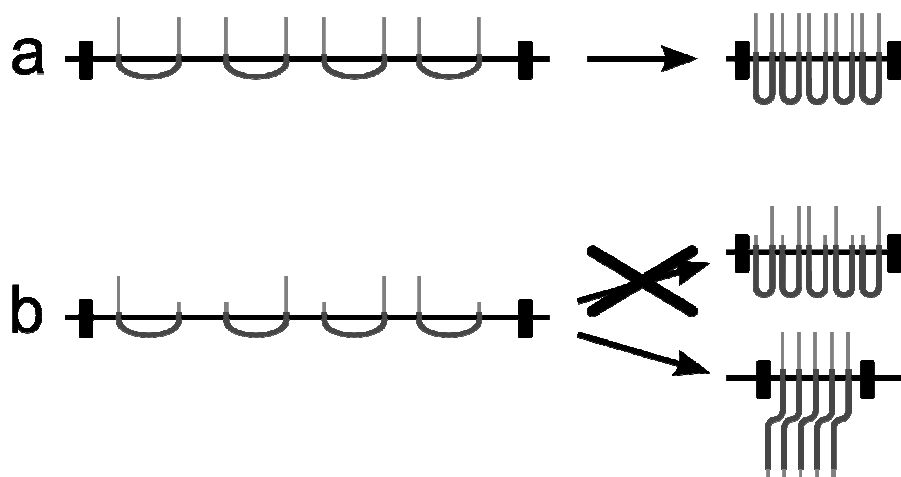
The results from the Langmuir isotherms and CD measurements give a clear picture for the conformation of peptides **3-5** and **8** and **9**. For these molecules a molecular area was extrapolated of just over double the size of a stearic acid molecule. The first rise of the curve compared favorably with DSPC, a neutral amphiphile with two stearyl tails, demonstrating that the molecular area occupied by these peptides is mainly determined by the lipophilic alkyl chains. Therefore, the peptide part of the amphiphile must be able to adopt such a conformation which facilitates a close packing of the alkyl tails in a similar fashion as for the model phospholipid DSPC (figures 4 and 5). The  $\beta$ -turn fold of the GANPNAAG sequence allows for such a close-packed fold and a molecular model of compound **5**, as shown in figure 7, indicates that such a fold is indeed feasible. This is substantiated by the observed CD spectrum of the transferred monolayers showing a  $\beta$ -sheet character of the folded peptide amphiphiles.



**Figure 7.** A) Spacefilling model of **5** showing the peptide in a  $\beta$ -turn conformation, with the aliphatic tails closely packed. B) Model of the peptide part of the compressed amphiphile indicating the intramolecular hydrogen bonds stabilizing the  $\beta$ -turn of the peptide.

Symmetrically functionalized GANPNAAG peptides with either C12 (**2**) or C6 (**1**) alkyl tails are water soluble to such an extent that they do not form stable monolayers. However, CD spectroscopy on transferred monolayers still shows the presence of a  $\beta$ -turn conformation for **2**.

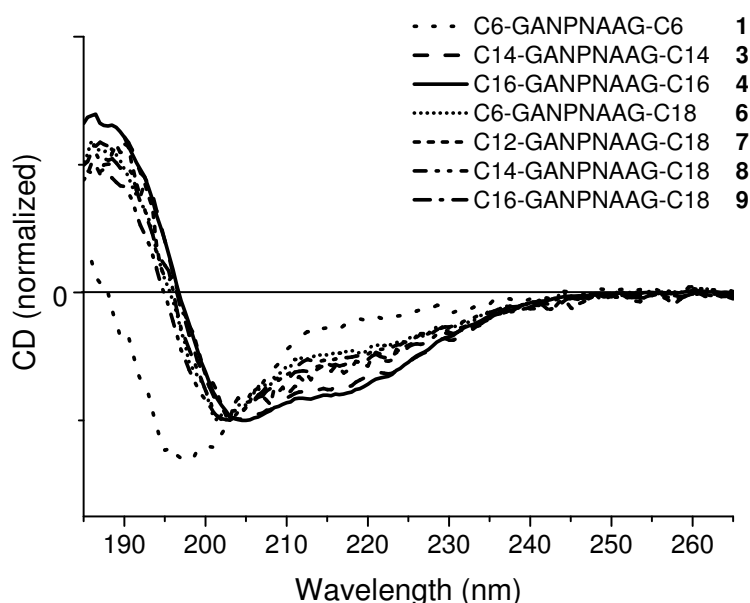
The Langmuir isotherm and CD measurement results of the asymmetric peptides **6** and **7**, with C6 and C12 N-terminal alkyl chains respectively, indicate a different type of conformation. First, in contrast to symmetrical peptides **1** and **2** stable monolayers were obtained for these compounds with a higher average area per molecule than the more hydrophobic compounds (**3-5**, **8**, **9**). This indicates that these amphiphilic peptides are anchored at the air-water interface by the hydrophobic C18 tail, but that the size of the area per molecule that is taken up by these peptides is increased due to the water solubility of the C6 and C12 alkyl tails. Moreover, the CD spectra of monolayers of these compounds at 20 Å<sup>2</sup> showed an intense peak at 200 nm, which was similar to the signal of the C16- and C18-GANPNAAG-OH fibrils described in chapter 4. All of these results can be explained by an unfolding of the peptide structure upon compression, resulting in immersion of the shorter alkyl tail in the water subphase, while the C18 tail remains in the air (figure 8).<sup>40</sup> The compression of the monolayer might have imposed an alignment of the peptides, which contributes to the strong CD signal.<sup>41</sup>



**Figure 8.** Langmuir monolayer experiments: schematic overview of the effect of compression on different amphiphilic peptides. A) Compression of amphiphilic peptides with tails of C14 or longer results in a stable monolayer in which the peptide is folded in a  $\beta$ -turn and the average area per molecule is determined by the hydrophobic tails. B) Compression of a peptide with a strongly hydrophobic alkyl tail and a more soluble short tail (**6**, **7**) does not result in a stable  $\beta$ -turn conformation. The hydrophobic tail anchors the amphiphile at the interface, while the shorter tail is forced into the aqueous phase, resulting in a monolayer where the area per molecule is determined by the peptide rather than the hydrophobic moieties.

### 5.3.2 Stabilization in liposomes

CD spectroscopy was also used to examine the folding of lipopeptides **1-5** mixed in liposomes of DSPC (C18). CD spectra of peptide **1** with two C6 tails in the presence of DSPC liposomes showed a minimum at 200 nm, indicative of a random coil conformation of the peptide. In contrast the CD spectra of peptides **2-5**, with tails increasing from C12 to C18 showed the presence of a  $\beta$ -sheet conformation (figure 9). Remarkably, even peptide **2** with two C12 tails, which did not form a stable monolayer, showed sufficient interaction with the DSPC vesicle to stabilize its  $\beta$ -turn conformation. Variation in length of the hydrophobic tails of these peptides did not result in a reproducible difference on the CD spectra. Therefore, the interaction between these tails and the DSPC bilayer, which depends in part on the match in length between these hydrophobic domains did not influence the folding characteristics of the peptides.



**Figure 9.** CD spectra of GANPNAAG amphiphiles incorporated in DSPC liposomes.

In order to determine the influence of the number of carbon atoms in the phospholipid tails that form the bilayer on the folding of the lipopeptides, the peptide amphiphiles were also mixed with DPPC and DMPC with C16 and C14 hydrophobic tails respectively. These liposome preparations showed the same folding as the peptides in the DSPC liposome;

peptide **1** with the C6 tails showed a random coil conformation, while the spectra of the other peptides (**2-5**) indicated a  $\beta$ -turn conformation.

Asymmetric lipopeptides **6-9** were also first incorporated in vesicles of phospholipid DSPC. There was no difference in observed conformation between the various asymmetric lipopeptides or between the symmetric and asymmetric peptides. In all cases  $\beta$ -turn characteristics were observed. Incorporation of the asymmetric peptides in DPPC or DMPC liposomes did not influence the folding of the peptides either. In case of peptide **1** the two C6 tails did not interact with liposomes and the  $\beta$ -turn of the peptide was not stabilized. Peptide **6** containing a C-terminal C18 and an N-terminal C6 chain, however did show stabilization of the  $\beta$ -turn, as indicated by the CD spectrum. Interaction between the C-terminal C18 tail and the phospholipid vesicle places the C6 tail close enough to the bilayer to allow hydrophobic interaction and folding to take place.

No effect was observed of alkyl tail length on the stabilization of peptide structure at room temperature, as long as the tails were longer than C6. Therefore, the vesicles of phospholipids with incorporated lipopeptides were heated in the CD-spectrophotometer in order to determine whether the stabilization of the peptide could be influenced by temperature. Still, there was little change in the CD spectrum on heating the sample to 90 °C, the intensity of the minimum at 200 nm lowered by 10 percent, but this change was rapidly and completely reversible on cooling.

## 5.4 Conclusion

Introduction of alkyl tails longer than C12 at both termini of the  $\beta$ -turn forming GANPNAAG peptide sequence stabilizes these turns in monolayers at an air-water interface. Furthermore, incorporation of the peptides in a dynamic phospholipid scaffold is a robust way to stabilize the conformation of this peptide sequence in a  $\beta$ -turn. The scaffold stabilizes the  $\beta$ -turn fold of peptides even when the tails are not hydrophobic enough to stabilize these structures in a monolayer. No effect was observed of the matching of hydrophobic domains of peptide and liposome on the stabilization of the turn. However, the peptide is short and the presence of the proline results in a predisposition for a turn. Furthermore, incorporation of the tails of both termini in the bilayer scaffold will result in the close proximity of both endgroups in the bilayer. This means that the peptides are pre-organized to form the  $\beta$ -sheet hydrogen-bonding pattern which in turn stabilizes the  $\beta$ -turn conformation. The influence of both

hydrophobic and hydrogen bond interactions probably contributes to the robustness of the stabilization.

## 5.5 Experimental

<sup>1</sup>H-NMR spectra were measured on a Varian Inova 400 MHz NMR spectrometer. MALDI-TOF spectra were measured on a Bruker Biflex II mass spectrometer, 2,5-dihydroxybenzoic acid (DHB) was used as a matrix. Peptides were synthesized on a Labortec SP4000 or a Labortec SP640 semi-automatic peptide synthesizer. Peptide couplings were followed to completion using the Kaiser test<sup>42</sup>.

### **C<sub>n</sub>H<sub>2n+1</sub>CO-Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly-NC<sub>m</sub>H<sub>2m+1</sub> 1-9**

Peptides were synthesized using a standard Fmoc solid phase synthesis protocol on an aldehyde resin, the C-terminal alkyl tail was introduced as described in chapter 2. Acylation of the N-terminus was achieved by a standard peptide coupling of the corresponding n-alkylcarboxylic acid. The resin was washed using DMF, DCM, methanol, DCM and diethylether. After drying, the products were cleaved from the resin by treatment with TFA/H<sub>2</sub>O (95/5) during three hours. After cleavage the peptides were purified by precipitation in diethylether, followed by silica column chromatography (chloroform/methanol/water; 65/24/4). The peptides were characterized using <sup>1</sup>H-NMR spectroscopy and MALDI-TOF mass spectrometry.

Typical analysis: **1** C<sub>5</sub>H<sub>11</sub>CO-Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly-NC<sub>6</sub>H<sub>13</sub>

Resin loading: 0.48 mmol g<sup>-1</sup> (65 %). <sup>1</sup>H-NMR (DMSO-d<sub>6</sub>) δ (ppm): 8.25 (d, 1H), 7.95 (t, 1H), 7.87 (t, 1H), 7.87 (d, 1H), 7.87 (d, 1H), 7.75 (d, 1H), 7.64 (s, 1H), 7.57 (d, 1H), 7.54 (t, 1H), 7.23 (s, 1H), 7.11 (s, 1H), 6.94 (s, 1H), 4.72 (q, 1H), 4.39 (q, 1H), 4.26 (qu, 1H), 4.22 (dd, 1H), 4.14 (qu, 1H), 4.12 (qu, 1H), 3.70 (m, 4H), 3.60 (m, 2H), 3.00 (q, 2H), 2.66 (d, 1H), 2.54 (d, 1H), 2.41 (d, 1H), 2.40 (d, 1H), 2.10 (t, 2H), 2.07 (m, 2H), 1.82 (m, 2H), 1.44 (qu, 2H), 1.35 (t, 2H), 1.22 (d, 6H), 1.20 (m, 18H), 1.12 (d, 3H), 0.82 (t, 6H)

Maldi-TOF mass spectra of peptides **1-9**

Compound	(M) <sup>+</sup> calc	(M+Na) <sup>+</sup> observed	(M+K) <sup>+</sup> observed
<b>1</b>	851.49	873.91	
<b>2</b>	1019.67	1042.10	
<b>3</b>	1075.74	1098.09	
<b>4</b>	1131.80	1154.40	
<b>5</b>	1187.86	1200.42	
<b>6</b>	1019.67	1042.12	1058.17
<b>7</b>	1103.77	1126.49	1152.55
<b>8</b>	1131.80	1155.21	1170.20
<b>9</b>	1159.83	1182.55	1198.70

## Langmuir monolayer experiments

Langmuir-trough monolayer experiments were performed at room temperature on a thermostated (20.0 °C Lauda RM6) double barrier R&K trough (6 × 25 cm). Peptides were dissolved to a final concentration of 1 mg mL<sup>-1</sup> in a mixture of chloroform and methanol (9/1 v/v), heated and sonicated in a closed vial. The solution (10–50 µL) was spread on the water subphase and the compression isotherm was recorded after 15 minutes.

## Liposome preparation

Peptides **1–10** were dissolved in a mixture of chloroform and methanol (9/1) to a final concentration of 1 mg mL<sup>-1</sup>. Solutions were prepared of di-alkyl-phosphatidylcholine (DMPC, DPPC or DSPC) in the same solvent mixture to a concentration of 10 mg mL<sup>-1</sup>. Mixtures were made of the peptide and phospholipid solutions in glass vials to a final concentration of 10% lipopeptide by weight. The solutions were dried by a slow nitrogen-flow and subsequent exposure to vacuum.

The samples were suspended in water to a total amphiphile concentration of 1 mg mL<sup>-1</sup> (i.e. a lipopeptide concentration of 0.1 mg mL<sup>-1</sup>) by heating to 60 °C for 1 hour with intermittent vortexing. The suspensions were homogenized by sonication at 60 °C for an hour.

## CD measurements

CD spectra were measured in a jasco 810 spectrophotometer fitted with a peltier temperature control unit (jasco PT-423 s/1). Samples were measured at 25 °C in a 1 mm quartz cell. Soluble peptides were dissolved in water to a concentration of 0.1 mg mL<sup>-1</sup>. Liposomes with lipopeptides were used as prepared.

## 5.6 References

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# 6

## **Amphiphilic peptides in a biologically active conformation**

*Alkyl functionalized Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly (GANPNAAG) peptides based on the CS protein of the malaria parasite were either bound in the lipid bilayer of a liposome or conjugated to a KLH carrier protein. The alkyl tails were introduced with the aim of using hydrophobic interactions to stabilize the sequence in the native  $\beta$ -turn conformation while being presented to the immune system. The stabilized  $\beta$ -turn peptide presented at the surface of a liposome induced the formation of antibodies when injected into rabbits, albeit at a low titer. Conjugates to KLH induced the formation of antibodies recognizing the peptide sequence as well. However, selectivity of the antibodies for the  $\beta$ -turn conformation could not be demonstrated*



## 6.1 Introduction

In this chapter the biological activity is examined of amphiphilic derivatives of the Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly (GANPNAAG) peptide sequence, by way of their immunogenic capabilities. This peptide sequence is derived from the circumsporozoite (CS) surface protein of the malaria parasite *Plasmodium falciparum*.<sup>1,2</sup> The CS protein is one of the main surface proteins of the sporozoite stage of this malaria parasite and contains 43 repeats of the Asn-Ala-Asn-Pro (NPNA) sequence.

In order to mimic biologically active domains of the native protein a model system not only has to encompass the amino acid sequence, but also the folding of the peptide has to be established.<sup>3,4</sup> Furthermore, haptens, immunogenic small molecules such as peptides, usually do not cause an efficient response of the immune system, although they are capable of interacting with antibodies. In order to elicit a response these small molecules need to be connected to a larger structure. For this purpose haptens are often conjugated to a carrier structure, such as polymers,<sup>5</sup> proteins<sup>6,7</sup> or liposomes.<sup>8</sup>

Robinson and coworkers have shown that the GANPNAAG peptide turn can be stabilized by introducing a covalent scaffold. This stabilized turn is the biologically significant structure, as antibodies raised against the stabilized turn sequence also recognized the sporozoite.<sup>9,10</sup> The cyclized epitope was functionalized with hydrophobic tails and incorporated in so-called immunopotentiating reconstituted influenza virosomes (IRIV) to present efficiently multiple copies of the epitope to the immune system.<sup>11</sup>

Instead of using a covalent approach to stabilize the hairpin structure of NPNA, as described by Robinson, a non-covalent method using hydrophobic interactions was envisaged, since in the previous chapter we demonstrated that the GANPNAAG peptide can be stabilized in a  $\beta$ -turn conformation via the attachment of hydrophobic tails to the N-terminus and C-terminus.

Two carrier options for the presentation of the non-covalently stabilized peptide were selected: liposomes and a carrier protein. The liposomal system described in Chapter 5 not only stabilized the conformation of the Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly sequence in a biologically relevant  $\beta$ -turn, but also resulted in a multivalent presentation of the peptides.

The ligation of haptens to proteins is a common tool to prepare immunogenic samples, and the ligation chemistry is well studied and readily compatible with peptide synthesis. Therefore, it was interesting to examine whether the stabilizing effect of hydrophobic tails could be used in conjunction with a carrier protein. As a scaffold Keyhole Limpet

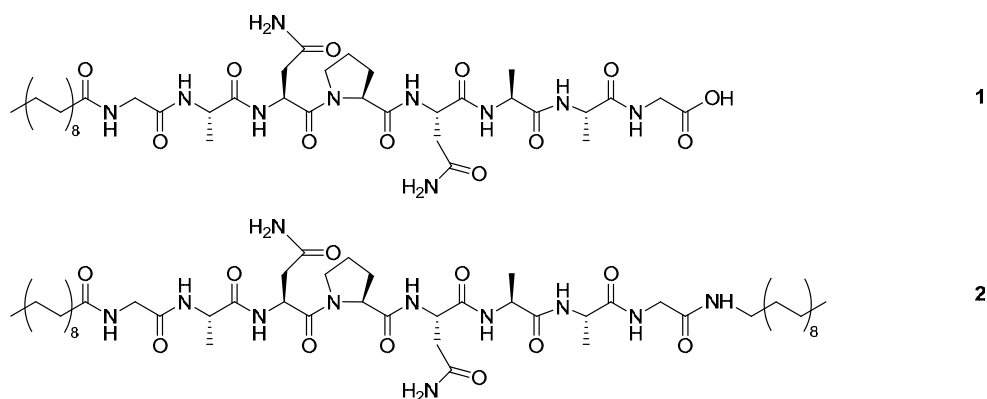
hemocyanin (KLH) was selected, a widely used commercially available carrier protein.<sup>14,15</sup> This non-mammalian protein is highly immunogenic, and provides many lysine residues that can be readily functionalized.

The preparation of GANPNAAG containing immunogenic liposomal solutions and peptide-KLH conjugates is described in this chapter. Moreover, the interaction between antibodies raised against these systems and model peptides containing the GANPNAAG sequence is examined. An immunoblotting technique is used to evaluate the level and specificity of antibodies in serum. The interactions are further examined using surface plasmon resonance (SPR).

## **6.2 Synthesis**

### **6.2.1 Peptides**

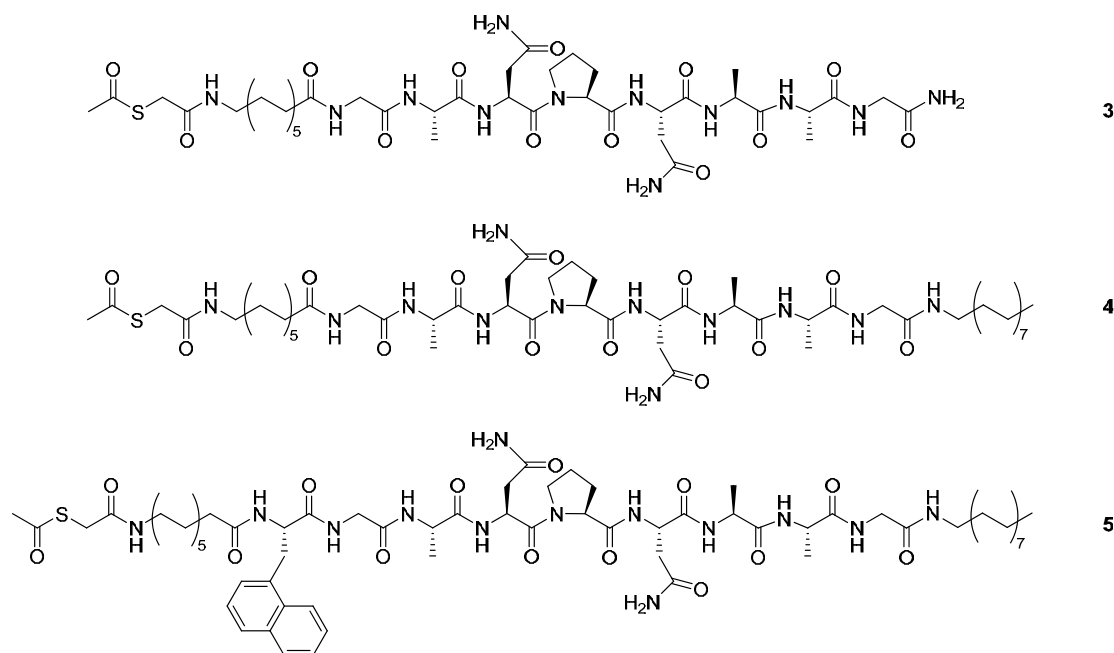
Two sets of peptides were synthesized for the examination of the biological activity of the hydrophobically modified GANPNAAG sequence. The first set (**1** and **2**, figure 1) was used for insertion of amphiphilic peptides into the bilayer membrane of a liposome. As previously published and described in chapter 5, incorporation of the GANPNAAG sequence functionalized with two hydrophobic tails in a liposome stabilized the peptide in a  $\beta$ -turn conformation.<sup>16</sup> In contrast, the interaction between a singly alkylated amphiphile and a liposome resulted in a random coil conformation for the peptide. The system with C18 functionalized peptides and the C18 distearoyl phosphatidylcholine (DSPC) liposomal scaffold was chosen for the immunogenicity tests of these amphiphilic peptide structures. These peptides were synthesized on a solid phase support. Monoacylated peptide C18-GANPNAAG **1** was synthesized on a Wang resin as earlier described in chapter 4. The dialkyl C18-GANPNAAG-C18 **2** was synthesized on an aldehyde resin as described in chapter 5.



**Figure 1** Structure of the peptides which were incorporated in DSPC liposomes in order to form an immunogenic system.

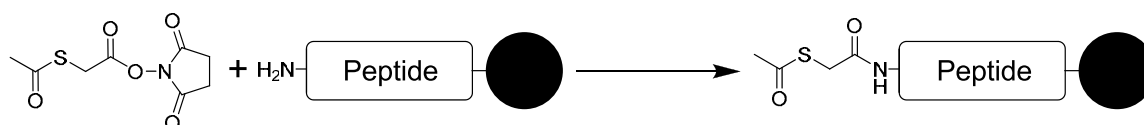
For the second approach peptides needed to be synthesized that contained a thiol moiety for coupling to the KLH carrier protein (figure 2). Two peptides were synthesized, one with a tail at the N-terminus of the peptide (**3**); the second with alkyl tails on both N- and C-termini (**4**), which were expected to stabilize the peptide in a  $\beta$ -turn conformation. In both cases the alkyl tail at the N-terminus was  $\omega$ -aminododecanoic acid, which allowed for the introduction of a protected sulfhydryl group at the end of the alkyl tail. Peptide **4** contained a C16 tail on the C-terminus, which in combination with the C12 tail should be able to stabilize the  $\beta$ -turn conformation as was shown in chapter 5.

Peptides **3** and **4** were prepared on the solid phase as described previously. Peptide **3** was prepared on a Breipohl resin as described in chapter 4, while peptide **4** was synthesized on an aldehyde resin after introduction of hexadecyl-amine following the procedure in chapter 5. After the deprotection of the final glycine, Fmoc-aminododecanoic acid was introduced to the N-terminus using standard peptide coupling chemistry. Finally, a protected thiol was introduced using the NHS activated ester of aceto-thioacetic acid (SATA, figure 3). This moiety can be readily deprotected before use yielding a free sulfhydryde, which can be quickly used before oxidation results in dimerization.



**Figure 2.** Peptides synthesized for conjugation with a carrier protein. The N-terminal tail is modified with a protected sulfhydryl (SATA) group

In order to be able to observe whether the hydrophobic doubly alkylated peptide is effectively coupled to the carrier protein a model peptide was synthesized containing a fluorescent label (**5**). After the introduction of the final glycine an Fmoc protected  $\beta$ -(1-naphthyl)-alanine was introduced. Subsequently, the synthesis was completed as described above.

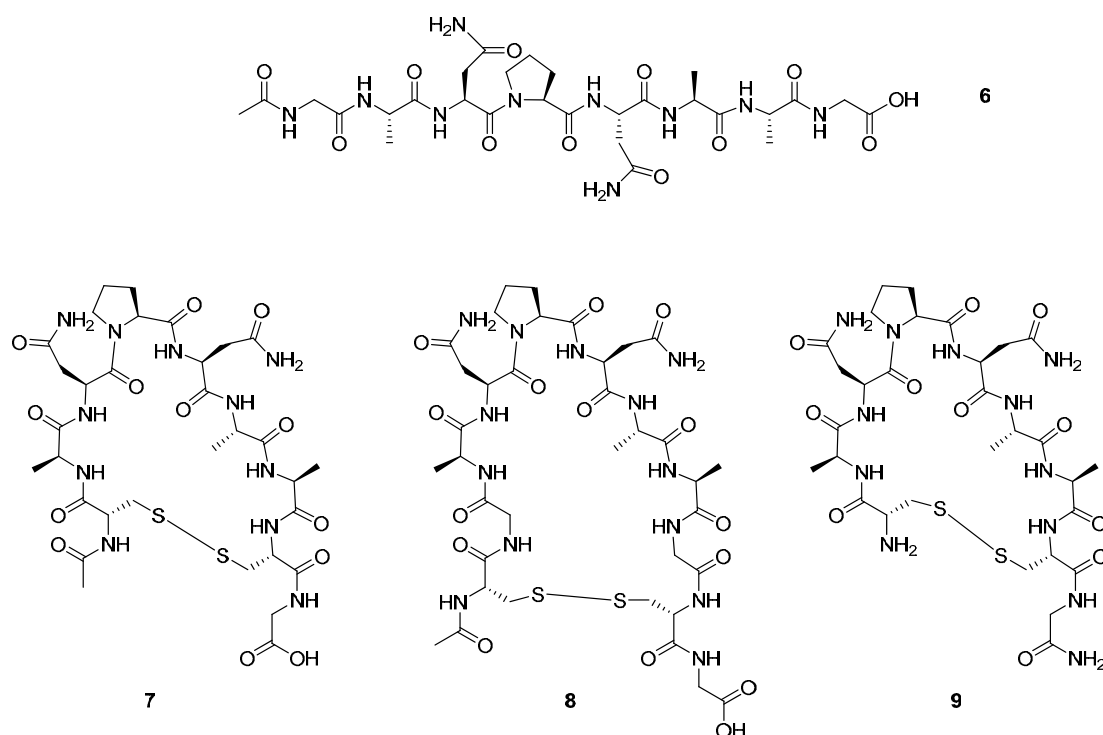


**Figure 3.** Conjugation of SATA to the free N-terminus of a peptide on a solid phase.

In addition, model peptides were synthesized with a fixed conformation (figure 4). These peptides were necessary to check the presence and selectivity of raised antibodies. The linear water soluble GANPNAAG **6** attains a random conformation, and was synthesized as described in chapter 4. Conformationally constrained peptides were obtained by introducing a cysteine disulfide bridge. Cysteines were introduced in the GANPNAAG sequence, resulting in two stabilized structures. Firstly, cysteines replaced the glycines in the peptide, yielding a stabilized  $\beta$ -turn (**7**, **9**). Secondly, cysteines were introduced flanking the GANPNAAG sequence, resulting in a loop with some more conformational freedom (**8**).

The cysteine containing peptides were synthesized and cyclized on the solid support. Peptides **7** and **8** were synthesized on a Wang resin, standard Fmoc protocols were followed.

The first cysteine introduced was protected with a trityl group while the second was protected with an acetamidomethyl moiety which has been found to promote intramolecular cyclization.<sup>17</sup> After the final acylation, the resin was suspended in DMF and nitrogen was led through the suspension. Subsequently, the resin was treated with iodine resulting in deprotection of the cysteines and subsequent ring-closure. Finally, the peptides were cleaved from the resin using TFA/water (95/5), lyophilized from water and characterized using NMR and mass spectrometry. Peptide **9** with an amide C-terminus was synthesized on a Breipohl resin, which allowed us to make use of the free N-terminal amine to introduce the peptide to the surface of an SPR chip. Also this peptide was treated with iodine after removal of the final Fmoc protecting group, before its subsequent cleavage from the resin.



**Figure 4.** Peptides with various stabilized conformations that were synthesized as model compounds for binding studies. Linear peptide **6**, peptide **7** with a stabilized  $\beta$ -turn, peptide **8** with a stabilized loop and peptide **9** with a stabilized  $\beta$ -turn and a free amine for conjugation to a surface.

### 6.2.2 Preparation of liposomes

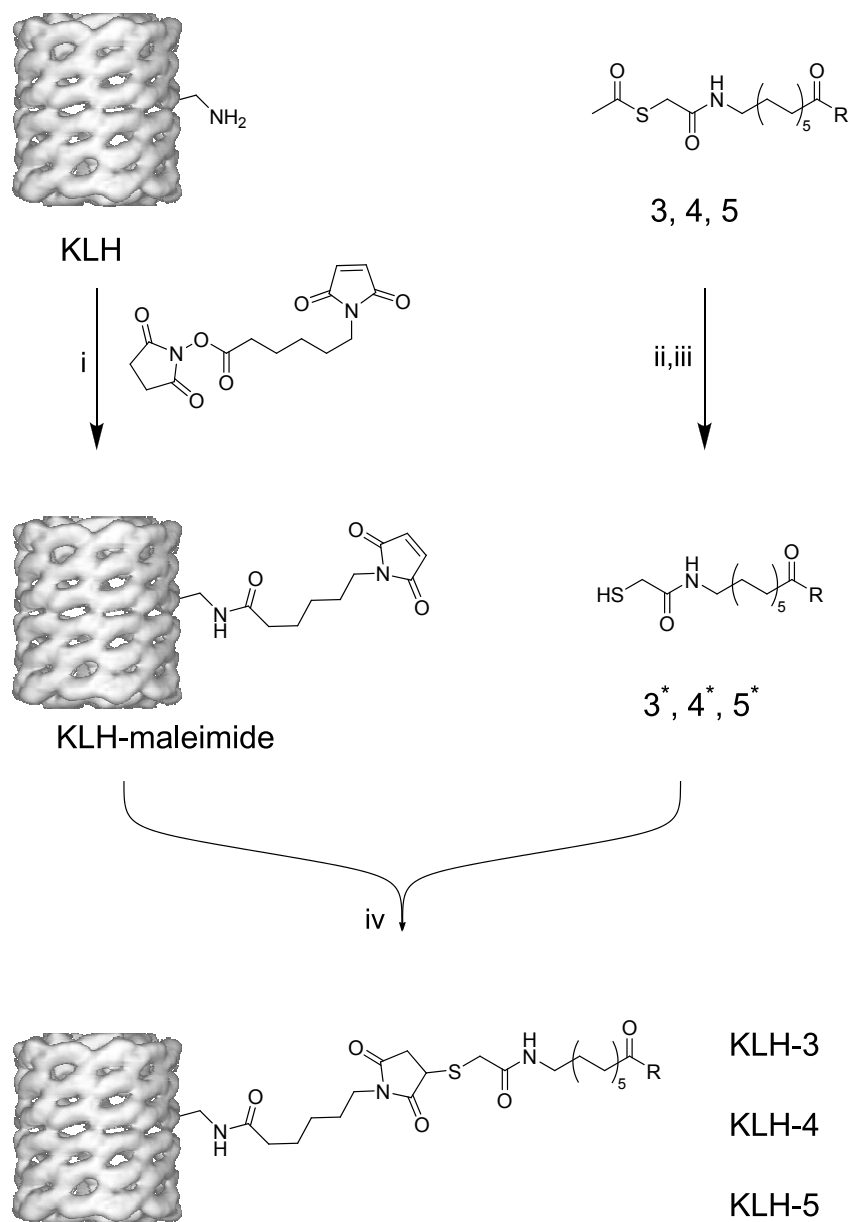
The general procedure to obtain functionalized liposomes described in chapter 5 was also employed to prepare DSPC liposomes containing **1** and **2** for the immunogenicity experiments. The samples were prepared to a final concentration of 10 percent of peptide by weight containing 0.2 mg peptide in 500  $\mu$ L of physiological PBS buffer. To minimize the effect of any residual organic solvents the mixture of peptide amphiphile and phospholipid

was dried thoroughly in vacuo for at least 18 hours. Furthermore, the liposomes were freshly prepared shortly before each injection.

Since the buffer was a new component for the liposomal system, the conformation of the peptides in the liposomes was checked using CD spectroscopy. Liposome solutions were diluted to a final peptide concentration of  $0.2 \text{ mg}\cdot\text{mL}^{-1}$ . The CD spectra showed that the conformation of the peptides in PBS was identical to the conformation in pure water as has been described in chapter 5.

### 6.2.3 Ligation to KLH

Keyhole limpet hemocyanin (KLH) was used as the carrier protein for conjugation of the amphiphilic peptides **3-5**.<sup>14</sup> This widely used large carrier protein stimulates a response by the immune system and can be readily functionalized. The peptides were coupled using an established protocol (scheme 1). First the amines of KLH were functionalized using 6-(maleimido)hexanoyl-N-hydroxysuccinimide (MHS) introducing maleimides on the protein. Subsequently, the acetyl protecting group of the thiol moiety of peptides **3-5** was removed by treatment with a concentrated NaOH solution. After quenching this reaction via the addition of acetic acid, the peptide was immediately added to the protein solution and allowed to react at room temperature. Finally, the reaction mixture was purified by dialysis, resulting in 10 mL of buffer containing 4 mg of protein. This solution was divided in aliquots for immuno-injection. For the initial injections the concentration of protein was doubled by lyophilization and subsequent re-dissolution. Care had to be taken not to mix the KLH protein solution too violently since this could lead to precipitation.



3: R = -Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly-OH

4: R = -Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly-NHC<sub>16</sub>H<sub>33</sub>

5: R = -1-naphtylalanine-Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly-NH<sub>16</sub>H<sub>33</sub>

**Scheme 1.** Conjugation procedure of ATA labeled peptides to KLH, by functionalization of KLH with maleimide and subsequent coupling with a free thiol on the peptide. At room temperature; i) pH 7.2, 5 min subsequent 2 times dilution with pH 6.0 Phosphate Buffer; ii) DMF, methanol and 8M NaOH, 0.5 min; iii) quenching with acetic acid; iv) 4 hours reaction. Subsequent twofold dilution and dialysis against pH 6.9 50 mM PBS.

Since the alkyl modified hydrophobic peptides **3-5** were insoluble in water they were dissolved in DMF for the first steps of the ligation procedure. Although no precipitation was observed after addition of the peptides to the KLH solution it was conceivable that the ligation reaction could be hindered by their hydrophobicity. To investigate this, peptide **5** containing a fluorescent probe was ligated to KLH using the procedure described above. After dialysis UV and fluorescence spectra were recorded in order to determine the presence of peptide. The measurements showed a fluorescent signal, confirming the effectiveness of the ligation reaction. Since the naphtyl containing model peptide **5** is more hydrophobic than peptides **3** and **4**, it was expected that the ligation of these peptides to KLH had also proceeded efficiently.

### **6.3    *Biological activity procedures***

#### **6.3.1    Liposomes**

In order to evaluate the immunogenicity of the liposomal systems rabbits were injected (in duplo) with DSPC liposomes containing either C18-GANPNAAG-C18 (**2**) or C18-GANPNAAG-OH (**1**). Sera of these rabbits were tested for the presence of antibodies that could recognize linear GANPNAAG **6** or the cyclic model peptides **7** or **8** (vide infra). Because the tests showed that after the first injections no immediate immune response was elicited. Therefore, booster injections were injected with an interval of 3 weeks up to a total of 10 times.

#### **6.3.2    KLH conjugates**

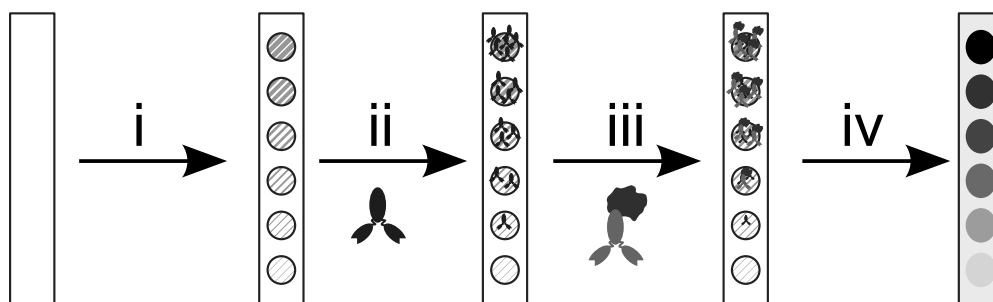
KLH conjugates were injected following a two phase protocol. In the first step conjugates were injected into the lymph nodes, with addition of Freund's adjuvant. Subsequent booster injections served to stimulate the immune system further.



## 6.4 Analysis of immune response

### 6.4.1 Spot test

The first evaluation of the sera for the presence of antibodies recognizing the GANPNAAG hapten was based on a spot test (figure 5). Model peptides for different GANPNAAG conformations (6-8) were non-covalently immobilized at a range of densities on a nitrocellulose strip (i). Aspecific binding was prevented by blocking the remaining surface with milk-powder. These treated strips were incubated with diluted crude serum, and excess material was subsequently washed away (ii). The strips were subsequently developed by incubation with general HRP-labeled anti-rabbit antibodies (iii). Finally, reagents were added in order to visualize the antibodies using chemiluminescent detection (iv).



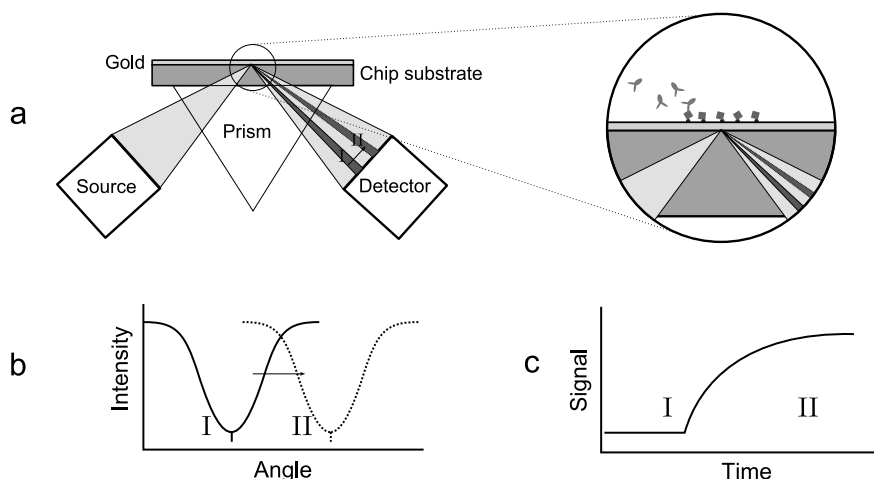
**Figure 5.** Spot test for the determination of the presence of antibodies. i) immobilization of model peptides and subsequent blocking by milk; ii) incubation with crude serum, followed by washing; iii) incubation of HRP-labeled antibody, followed by washing; iv) addition of reagents, followed by detection on film.

This methodology yielded preliminary information on the presence and selectivity of antibodies in the serum. Furthermore, serum dilution and exposure during the chemiluminescent detection phase could be varied to detect differences in antibody concentration or binding strength.

### 6.4.2 SPR

To further examine the properties of the raised antibodies their interactions with model peptides were investigated using a surface plasmon resonance based biosensor (SPR), specifically a Biacore 2000 system. The principle of SPR can be described as follows (figure 6): a thin layer of gold reflects light, but at a specific angle the interaction between the light and surface plasmons results in absorption.<sup>18</sup> The absorption angle is dependent on the dielectric constant of the material above the gold surface. Binding of a molecule to an immobilized substrate on the gold surface changes the dielectric constant and is therefore

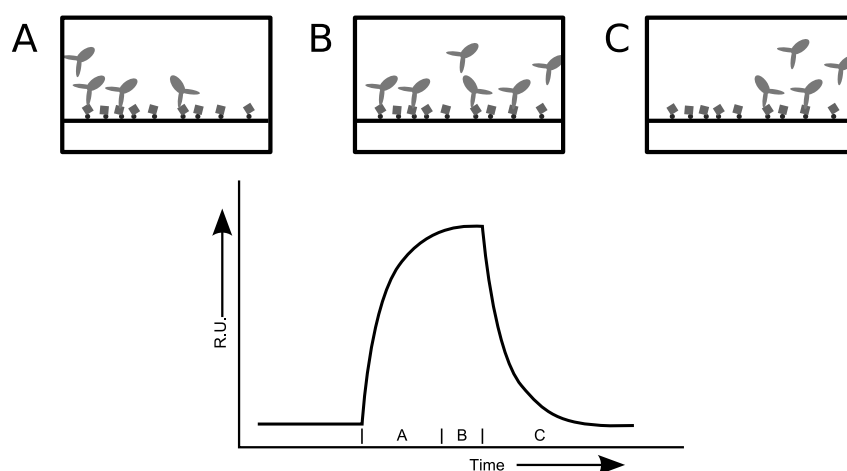
detectable as a change in the absorption angle. The change in dielectric constant is correlated to the mass of the binding species. In a flow cell, such as used in the Biacore system, this binding process can be monitored in time.



**Figure 6.** Schematic overview of SPR. A) Monochromatic polarized light is reflected from a gold film on the interface between the chip substrate and the analysis channel, resulting in an absorbance at a specific angle. B) Upon binding to the surface, a refractive index change leads to a change in the absorption angle. C) The change in angle can be followed in time, which is correlated to the amount of molecules bound to the surface.

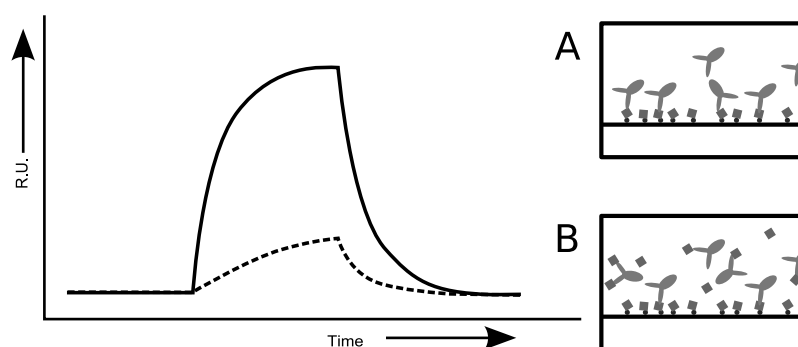
The Biacore system uses standardized chips, functionalized with carboxymethylated dextran. A single chip contains multiple, simultaneously addressable channels. This allows for an immediate correction of non-specific interactions between chip surface and solutes. Cystine stabilized peptide **9** was immobilized on the chip surface through its free amine at the N terminus, using a standard EDC – NHS mediated coupling. A reference channel was obtained by functionalization of the surface with amino-ethanol. This channel allowed for correction of the effects of aspecific interactions between solute and surface.

In two approaches the interactions between the antibodies in the sera and the immobilized peptide **9** were examined. In the first the effect of the concentration of the antibodies on binding was investigated (figure 7). The maximum response of the binding curves was expected to yield some more information on antibody concentration, while on- and off curves should give some data on selectivity and kinetics. Since SPR is capable of distinguishing between specific and aspecific interactions crude serum was used. The serum was diluted with PBS to a concentration at which a binding curve could be readily observed.



**Figure 7.** Schematic representation of an SPR binding curve, the shape of the curve is determined by association (A), reaching equilibrium (B) and finally dissociation (C) of antibodies to an immobilized hapten.

In a second approach the competitive effect of the presence of peptides in solution on the binding of antibodies was examined (figure 8). It is possible to introduce different peptides on the SPR chip, but variation in functionalization introduces further uncertainties in our system. Therefore, in order to obtain information on the specificity of the antibodies in the sera competition experiments were performed using a singly modified chip and different peptides in solution. Antibodies bound to a hapten in solution no longer bind to the surface, and varying concentrations of inhibitor in solution should reveal the differences in affinity to different conformations of the peptide of interest (figure 8). The surface was functionalized with a cystine stabilized loop **9**, as used before in the dilution experiments described above. Serum was diluted using PBS and from stock solutions either linear peptide **6** or cyclic peptide **9** were added. The resulting solutions contained serum with peptide at concentrations of 1 mM, 0.1 mM, 0.01 mM and no peptide at all.

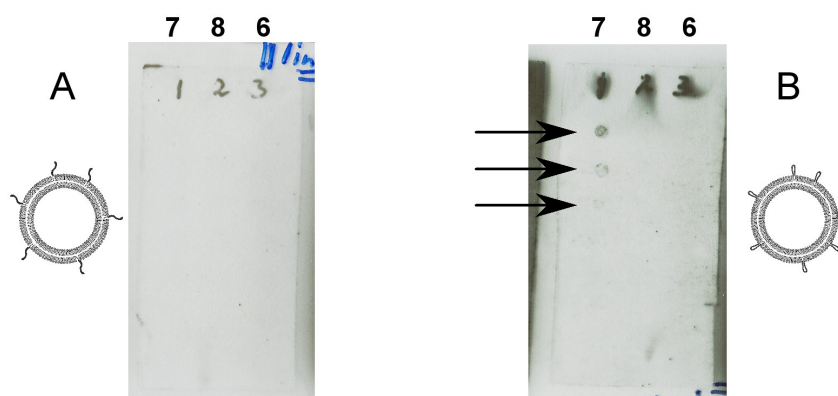


**Figure 8.** Schematic overview of an SPR binding competition experiment. Upon addition of a competing peptide to the serum the interaction with the immobilized hapten diminishes.

## 6.5 Results

### 6.5.1 Spot test

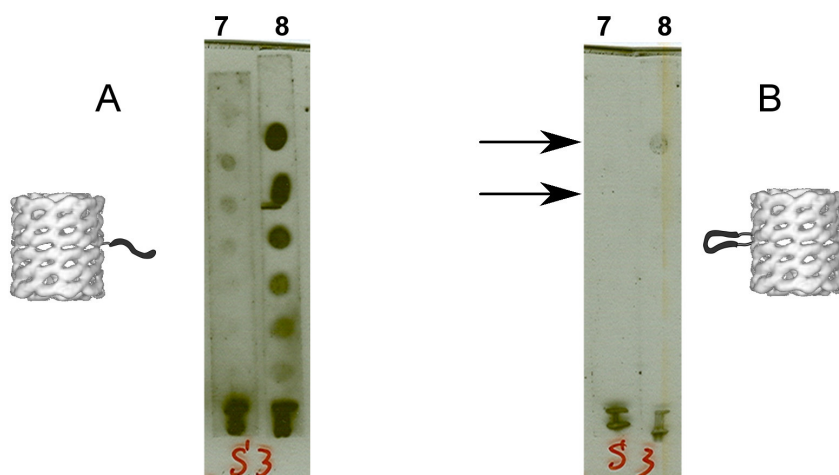
Using the spot test on the sera raised by exposure to the liposomes, antibodies were detected only in case of the DSPC vesicles functionalized with the stabilized  $\beta$ -turn of di-alkylated GANPNAAG (**2**) (figure 9). These antibodies specifically recognized the stabilized  $\beta$ -turn of model peptide **7**. However, the response was very weak and only observed for one of the two sera. Neither linear sequence **6** nor the more open loop of **8** was recognized by antibodies in this serum. No response was observed in either of the samples raised against the liposomes containing monoalkylated peptide **1**.



**Figure 9.** Spot-tests for the sera raised against liposomes containing GANPNAAG, the sera were tested for antibodies recognizing GANPNAAG in a  $\beta$ -turn (7), a loop (8) and as linear peptide (6) A) Response to the sera of liposomes containing singly alkylated 1. B) Response to the sera of liposomes containing doubly alkylated 2. Antibodies recognizing  $\beta$ -turn model peptide 7 are indicated with arrows.

Sera raised against KLH conjugates **KLH-3**, with the singly alkylated peptide, and **KLH-4**, with two hydrophobic tails on the peptide were tested against the stabilized  $\beta$ -turn of model peptide 7 and the more open loop of 8. The serum raised against linear peptide conjugate **KLH-3** (figure 10) contained mostly antibodies that recognized the open loop of cyclized peptide 8. The stabilized  $\beta$ -turn of peptide 7 was also recognized, but this response was significantly lower. The response of both samples raised against **KLH-3** was not identical. The response for the open loop (8) was similar, but one sample showed a markedly lower response to  $\beta$ -turn 7.

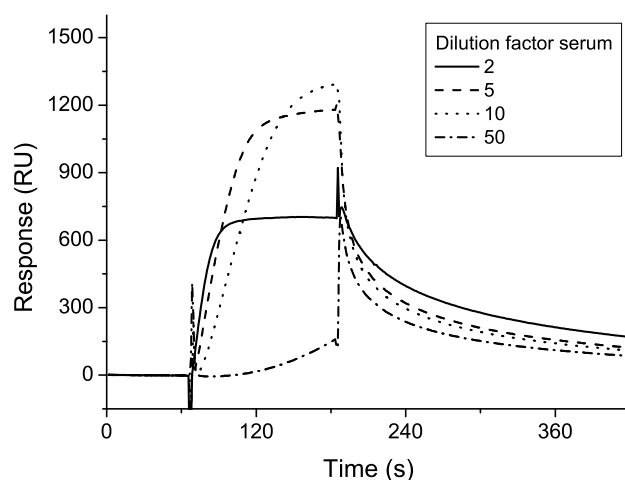
The titer of the sera raised against the conjugated di-alkyl peptide **KLH-4** was much lower than for the sera obtained from the experiment with **KLH-3**. Only antibodies were present that recognized model peptide 8. This response was only observed for one of the two sera, the titer of the second serum was too low to be observed using this test.



**Figure 10.** Results for spot-tests for the sera raised against KLH conjugates containing GANPNAAG. The sera were tested for antibodies recognizing GANPNAAG in a  $\beta$ -turn (7) or a loop (8) (figure 4). A) Antibodies recognizing the open loop of 8 were prevalent in the serum raised against **KLH-3**, B) Antibodies recognizing 8 were present at a low titer in the serum raised against **KLH-4** (indicated with arrows).

### 6.5.2 SPR

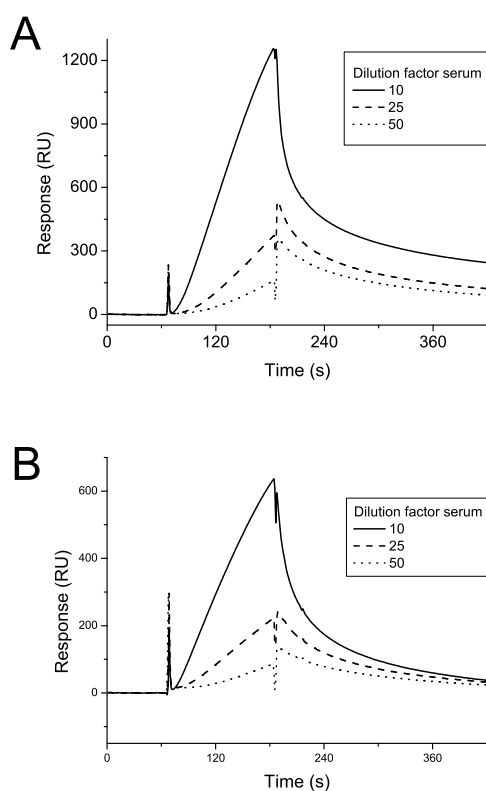
Of the liposomal sera only the spot-test positive sample raised against the stabilized  $\beta$ -turn (2) was tested using SPR (figure 11). Selective binding to the surface was only observed at high concentrations of serum. Upon increasing the dilution of the serum the shape of the binding curve indicated a slower binding process, *i.e.* the slope of the curve became less steep. At the same time the absolute response increased, indicating more specific binding. This apparent contradiction indicated an influence, possibly competition, of non-specific interactions of compounds in the serum. Competitive binding curves for this serum showed a higher inhibition by the cyclic peptide (9) than by the linear peptide (6) (figure 13).





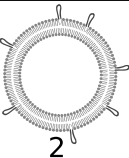


**Figure 11.** SPR curves demonstrating binding of antibodies in crude serum raised against liposome stabilized  $\beta$ -turn peptide **2**. Cyclic peptide **9** was immobilized on the chip surface and the serum was diluted with PBS.

Sera raised against conjugate **KLH-3**, with the singly alkylated peptide, resulted in slightly different responses depending on the exact serum that was tested. This was consistent with the spot tests which indicated a difference in titer in the two responses. Binding to the functionalized surface was lower in the presence of linear model peptide **6** than in the presence of cyclic **9**.

The sera raised against conjugate **KLH-4** had a lower response than was observed for **KLH-3**, which was consistent with the spot test. Both sera for **KLH-4** showed a similar response, this was unexpected since the spot-tests showed different titers. Competition experiments showed some influence of the presence of linear peptide **6** on the binding to the surface, while the effect of cyclic model peptide **9** in solution was negligible. The influence of either peptide was much lower for the sera of **KLH-4** than for either the sera of **KLH-3** or the liposomal sample.



**Figure 12.** SPR binding curves. Binding of antibodies in crude serum raised against conjugates **KLH-3** (A) and **KLH-4** (B). Cyclic peptide **9** was immobilized on the chip surface and the serum was diluted with PBS.

	 <b>9</b>	 <b>6</b>
 <b>2</b>	+++	++
 <b>KLH-3</b>	++	+++
 <b>KLH-4</b>	—	+

**Figure 13.** Schematic summary of the influence of peptides **9** or **6** in solution on binding of antibodies raised against liposomally stabilized  $\beta$ -turn **2**, and conjugates **KLH-3** and **KLH-4**. Competitive effect of peptides added in solution is qualitatively expressed by the signs, from (+++) for a strong competitive effect to (—) for no observed competitive effect.



## 6.6 Discussion

A clear difference is observed in the immunogenic response depending on peptide functionalization and carrier system. The spot test and SPR results indicate that the response to the KLH conjugates is higher than for the liposomal system. For the liposomal system both the long induction time before antibodies were detected and the low titer indicate a low immunogenicity. This can in part be explained by the fact that the immune system was not further stimulated by use of an adjuvant in conjunction with the liposome injections, due to incompatibility of standard adjuvants with liposomal systems. Furthermore, the stability and lifetime of the liposomes *in vivo* could have been low. It is conceivable they are rapidly cleared from the blood stream, resulting in only a short effective window of action.<sup>19</sup>

The instability of the liposomal system could also have been the reason for the difference in response to the mono-alkylated GANPNAAG **1** and the di-functionalized peptide **2** in the liposomal system. In chapter 4 it was shown that the singly alkylated C18-GANPNAAG **1** can form soluble fibrils in solution, while as observed in chapter 5 **2** is not soluble in water. It can be argued that this difference in solubility can have an effect on the dissolution of the liposomes *in vivo*. This can render the singly alkylated peptide **1** inactive while C18-GANPNAAG-C18 **2** retains some activity. The titer of the antibodies raised against the stabilized  $\beta$ -turn of **2** was low as was shown by both the spot test and the SPR studies; however, the selectivity of the binding was good.

In contrast to the liposomal system containing singly alkylated peptide **1** the analogous KLH conjugate **KLH-3** did show a response. This shows that in the right environment the conformationally free GANPNAAG sequence can stimulate the immune system. The spot test of the sera raised against **KLH-3** showed a decidedly larger response for the open loop of **8** than for the  $\beta$ -turn of model peptide **7** (figure 10). This difference was expected for the non-stabilized peptide. However, the SPR competition binding experiments showed a small difference in selectivity between the linear model peptide **6** and the  $\beta$ -turn of **9** (figure 14). However, since the chip surface was functionalized with a stabilized  $\beta$ -turn it is conceivable that mostly the antibodies in the serum that specifically recognize this conformation bind to the chip surface. This would be a specific subset of the antibodies present in the polyclonal mixture in the serum, resulting in a bias in the competition experiment. Since in this case the antibodies bound to the chip already readily recognize the  $\beta$ -turn conformation the competitive effect of  $\beta$ -turn peptide **9** would be larger than expected solely based on the spot test that tests the whole population.

The response of antibodies raised against di-functionalized conjugate **KLH-4** as determined using the spot test was low and the antibodies present recognized the open loop and not the  $\beta$ -turn (figure 10). However, SPR measurements showed that antibodies were present; moreover the competition experiments show that these antibodies bind better to an immobilized peptide on the chip than to a peptide in solution. The low titer and unexpected selectivity of the antibodies in these sera, especially in contrast to those raised against **KLH-3**, possibly indicate that in this conjugate the peptide is presented less effectively to the environment compared with the more hydrophilic conjugate **KLH-3**. It is conceivable that the increased hydrophobicity of the peptide at the terminus of the GANPNAAG sequence resulted in an interaction with the core of the carrier protein. Such an interaction could fold the hapten in a badly accessible conformation, which is not likely to be the desired stabilized  $\beta$ -turn. This could explain why the antibodies that were raised had specificity for the open loop of model peptide **8**. Furthermore, the low competitive effect of either model peptide on the binding of the antibodies in the serum raised against **KLH-4** could indicate that these antibodies were influenced by the close proximity of the protein surface to the epitope.

## 6.7 Conclusion

In this chapter it is shown that immunogenic constructs can be readily synthesized using peptides functionalized with hydrophobic tails. Peptide **2**, stabilized in a  $\beta$ -turn conformation by incorporation in DSPC liposomes, induced the formation of antibodies that preferably recognize the stabilized  $\beta$ -turn of the Asn-Pro-Asn-Ala sequence. This shows the effective presentation of this conformation to the immune system. However, the immune response was low. The non-stabilized peptide **1**, anchored to DSPC liposome through a single N-terminal C18 tail did not induce an observable immune response.

Hydrophobically modified peptides could be coupled to a carrier protein using standard bio-conjugation methodologies. The resulting conjugates gave rise to a response of the immune system. However, there was no strong correlation between conjugated peptide and the selectivity of the antibodies raised against the peptide. Moreover, the immune response of conjugate **KLH-4**, containing peptide **4b** with hydrophobic moieties at both C- and N-terminus was lower than for **KLH-3**. This, joined with the observed selectivity of the antibodies raises doubts on the usefulness of hydrophobic stabilization of peptide secondary structure in conjunction with a carrier protein.

## 6.8 Acknowledgements

The research presented in this chapter would not have been possible without the cooperation of several people. Prof. Stunnenberg, Anita Kaan and Josephine Jansen were invaluable for their contact with the animal lab, final preparation of injectable samples, collection and work with serum and the first evaluation for the presence of antibodies in the serum.

## 6.9 Experimental

### Materials and Methods

Synthesis of peptides **1** and **6** is described in Chapters 4, synthesis of peptide **2** is described in Chapter 5. MHS (N-succinidyl 6- maleidocaproate) was obtained from Pierce, KLH (inject KLH) was obtained from Pierce in the form of ampoules containing 20mg of KLH lyophilized from PBS buffer. CM5 chips and HBS-EP buffer for the SPR measurements were obtained from Biacore. Cyclic model peptides **7** and **8** were synthesized by Dennis Löwik, and analyzed by mass spectroscopy and TLC before use. SPR measurements were performed on a Biacore 2000. <sup>1</sup>H-NMR spectra were measured on a Varian Inova 400 MHz NMR spectrometer. MALDI-TOF spectra were measured on a Bruker Biflex II mass spectrometer, 2,5-dihydroxybenzoic acid (DHB) was used as a matrix. Peptides were synthesized on a Labortec SP4000 or a Labortec SP640 semi-automatic peptide synthesizer. Peptide couplings were followed to completion using the Kaiser test<sup>20</sup>.

### ATA-C12-Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly-NH<sub>2</sub> **3**

The peptide was synthesized using standard Fmoc peptide synthesis on a Breipohl resin.<sup>21,22</sup> Peptide coupling was achieved using 3 equivalents of Fmoc amino acid and diisopropylcarbodiimide (DIPCDI, 3.3 eq.) with N-hydroxy benzotriazole (HOBt, 3.6 eq.) in DMF. The resin was swollen in DMF for 20 minutes prior to use. The Fmoc-group was removed using piperidine in DMF (20% v/v, 3 times 6 minutes). Three equivalents of Fmoc-protected ω-aminododecanoic acid were dissolved in dichloromethane and 3.6 equivalents of HOBt (1M in DMF) were added. After mixing, 3.3 equivalents of neat DIPCDI were added. The solution was added to the resin and the resin was agitated for 18 hours; subsequently, the Fmoc group was removed. A solution of 2 equivalents aceto-thioacetic acid (SATA) in DMF was added to the resin, the suspension was agitated for 3 hours. After washing with methanol, dichloromethane and ether the resin was dried *in vacuo*. The peptide was cleaved from the resin by treatment with trifluoroacetic acid/water/triisopropylsilane/ethanedithiol (92.5/2.5/2.5/2.5, by volume) for 4 hours. After precipitation in diethyl ether the peptide was lyophilized from acetic acid.

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>) δ (ppm): 8.25 (d, 1 H), 8.01 (m, 3H), 7.94 (q, 1H), 7.87 (m, 4H), 7.75 (d, 1H), 7.64 (s, 1H), 7.57 (d, 1H), 7.44 (2, 1H), 7.24 (s, 1H), 7.12 (m, 5H), 6.95 (d, 2H), 4.72 (q, 1H), 4.55 (m, 1H), 4.39 (q, 1H), 4.26 (m, 1H), 4.22 (m, 1H), 4.14 (q, 2H), 3.70 (m, 2H), 3.64 (m, 2H), 3.60 (m, 3H), 3.52 (s, 3H), 3.14 (m, 1H), 2.9 (m, 3H), 2.66 (m, 1H), 2.54 (m, 1H), 2.41 (m, 2H), 2.05 (m, 4H), 1.95 (t, 1H), 1.82 (m, 4H), 1.44 (m, 2H), 1.35 (m, 2H), 1.20 (m, 18H). Maldi TOF Mass (C<sub>42</sub>H<sub>70</sub>N<sub>12</sub>O<sub>13</sub>S) (calc) 982.49 (M); (obs) 1004.78 ([M+Na]<sup>+</sup>), 1020.75 ([M+K]<sup>+</sup>)

### ATA-C12-Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly-NHC16 **4**

The C-terminal n-hexadecylamine tail for peptide **4** was introduced through reductive amination of 4-(4-formyl-3-methoxyphenoxy)butyryl aminomethyl resin,<sup>23</sup> as described in Chapter 2. This was followed by a standard Fmoc solid phase peptide protocol as described above for peptide **3**.

<sup>1</sup>H-NMR (DMSO-d<sub>6</sub>) δ (ppm): 8.28 (d, 1H), 8.05 (t, 1H), 7.95 (t, 1H), 7.85 (m, 2H), 7.75 (d, 1H), 7.65 (s, 1H), 7.55 (m, 2H), 7.24 (s, 1H), 7.12 (s, 1H), 6.95 (s, 1H), 4.54 (s, 1H), 4.48 (q, 1H), 4.27 (t, 1H), 4.20 (m, 1H), 4.10 (m, 2H), 3.75-3.55 (m, 4H), 3.50 (s, 1H), 2.95 (qu, 3H), 2.65 (m, 1H), 2.55-2.45 (m, 5H), 2.05 (m, 2H),

1.95 (m, 1H), 1.85 (s, 1H), 1.80 (m, 2H), 1.45 (m, 2H), 1.35 (m, 4H), 1.25 (m, 44H), 1.20 (m, 4H), 0.8 (t, 3H). Maldi TOF Mass ( $C_{58}H_{102}N_{12}O_{13}S$ ) (calc) 1206.74 (M); (obs) 1229.22 ( $[M+Na]^+$ ), 1245.20 ( $[M+K]^+$ )

### ATA-C12-1-Nal-Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly-NHC16 5

The C-terminal n-hexadecylamine tail for peptide **4** was introduced through reductive amination of 4-(4-formyl-3-methoxyphenoxy)butyryl aminomethyl resin,<sup>23</sup> as described in Chapter 2. This was followed by a standard Fmoc solid phase peptide protocol as described above for peptide **3**.

<sup>1</sup>H-NMR (DMSO- $d_6$ )  $\delta$  (ppm): 8.28-8.15 (m, 2 H) 8.12 (d, 0.5H), 8.01 (m, 1H), 7.94 (d, 0.5H), 7.87 (m, 3H), 7.75 (m, 2H), 7.64 (s, 1H) 7.60-7.5 (m, 3H), 7.35 (m, 1H), 7.22 (s, 1H), 7.12 (s, 1H), 6.95 (s, 1H), 4.72 (q, 1H), 4.55 (m, 1H), 4.39 (q, 1H), 4.26 (m, 1H), 4.22 (m, 1H), 4.14 (q, 2H), 3.70 (m, 2H), 3.64 (m, 2H), 3.60 (t, 3H), 3.52 (s, 1H), 3.14 (m, 1H), 3.00 (m, 3H), 2.66 (m, 1H), 2.54 (m, 1H), 2.41 (m, 2H), 2.05 (m, 1H), 1.95 (t, 1H), 1.82 (m, 4H), 1.34 (qu, 2H), 1.20 (m, 32H), 1.15 (m, 6H), 0.82 (t, 3H). Maldi TOF Mass ( $C_{71}H_{113}N_{13}O_{14}S$ ) (calc) 1403.83; (obs) 1427.20 ( $[M+Na]^+$ ), 1443.16 ( $[M+K]^+$ )

### Cyclic H-Cys-Ala-Asn-Pro-Asn-Ala-Ala-Cys-Gly-NH<sub>2</sub> 9

The peptide was synthesized on 1g of breipohl resin (0.52 mmol) using standard Fmoc solid phase synthesis protocols. For the first cysteine on the resin acetamidomethyl (Acm) was used as protecting group, the second cysteine was protected with a trityl (Trt) protecting group. Cyclization was performed after the removal of the final Fmoc protecting group. After the linear synthesis the resin was washed with DMF, DCM, methanol, DCM and diethylether and finally resuspended in DMF. Nitrogen was bubbled through the suspension for half an hour. Subsequently 3 grams of iodine dissolved in DMF was added and the mixture was agitated under a nitrogen flow for one hour. The resin was washed using DMF, a solution of ascorbic acid in aqueous DMF, methanol, DCM and diethylether. After drying, the peptide was cleaved from the resin by treatment with TFA/H<sub>2</sub>O (95/5) during three hours. After cleavage the peptide was purified by precipitation in diethylether, followed by silica column chromatography (chloroform methanol water; 65/24/4). The peptide was characterized using <sup>1</sup>H-NMR spectroscopy and MALDI-TOF mass spectrometry. <sup>1</sup>H-NMR (DMSO- $d_6$ )  $\delta$  (ppm): 8.7 (1H), 8.4 (1H), 8.15 (1H), 7.9 (3H), 7.7 (1H), 7.5 (1H), 7.4 (2H), 7.2 (2H), 7.1 (1H), 6.9 (1H), 4.8 (1H), 4.4 (2H), 4.2 (3H), 4.0 (1H), 3.6 (3H), 2.8 (1H), 2.6 (1H), 2.0 (2H), 1.8 (3H), 1.2 (9H) Maldi TOF Mass ( $C_{30}H_{48}N_{12}O_{11}S_2$ ) (calc) 816.30; (obs) 817.55 ( $[M+H]^+$ ), 839.53 ( $[M+Na]^+$ ), 855.52 ( $[M+K]^+$ )

#### Cyclic Ac-Cys-Ala-Asn-Pro-Asn-Ala-Ala-Cys-Gly-OH 7

Maldi TOF Mass ( $C_{32}H_{49}N_{11}O_{13}S_2$ ) (calc) 859.30; (obs) 860.49 ( $[M+H]^+$ ), 882.48 ( $[M+Na]^+$ ), 898.42 ( $[M+K]^+$ ).

#### Cyclic Ac-Cys-Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly-Cys-Gly-OH 8

Maldi TOF Mass ( $C_{36}H_{55}N_{13}O_{15}S_2$ ) (calc) 973.34; (obs) 996.49 ( $[M+Na]^+$ )

### Conjugation protocol (KLH-3, KLH-4)

A buffered (0.1 M phosphate, 0.15 M NaCl, pH 7.2) solution of KLH (10 mg mL<sup>-1</sup>) was obtained by dissolving an ampoule of lyophilized KLH (20 mg) in 2 mL of MilliQ. To the slightly opaque KLH solution 4.6 mg N-succinimidyl 6- maleidocaproate (MHS) dissolved in 125  $\mu$ L DMF was added. After mixing gently the reaction mixture was left at room temperature for 5 minutes. Subsequently, the activated KLH was diluted to 5 mL using a pH 6 phosphate buffer (0.05 M), resulting in a 4 mg mL<sup>-1</sup> protein solution.

Ata-conjugated peptide was deprotected immediately before coupling to the activated KLH to yield a free sulfhydryl group. First, 6 mg of peptide was dissolved in 140  $\mu$ L DMF. Next, 50  $\mu$ L methanol and 8  $\mu$ L of an 8M NaOH solution were added and the mixture was vortexed well. Finally, after 30 seconds 10  $\mu$ L of acetic acid was added to quench the reaction mixture.

The deprotected peptide was slowly added to 4 mg of the activated KLH, the reaction mixture was left for 4 hours at room temperature. Subsequently, the reaction mixture was diluted to 10 mL using a 0.05 M phosphate buffer at pH 6.9. The diluted reaction mixture was dialyzed for 2 hours, and again twice for 8 hours against a 0.05 M phosphate buffer (pH 6.9). A 500  $\mu$ L aliquot of the dialyzed solution was freeze dried and redissolved in 250  $\mu$ L MilliQ water for the initial injection in the immunization procedure. The remainder of the conjugate solution was divided in 900  $\mu$ L aliquots, stored at -20 °C and used for booster injections.

## Preparation of liposome-solutions

Solutions of liposomes were freshly prepared for inoculation of the rabbits. Peptide (C18-GANPNAAG-OH (1) or C18-GANPNAAG-C18 (2)) was dissolved in chloroform/methanol mixture (9:1, v/v) at a concentration of  $1 \text{ mg mL}^{-1}$ , and DSPC was dissolved in the same solvent to a concentration of  $10 \text{ mg mL}^{-1}$ . The amphiphiles were mixed by adding  $500 \mu\text{L}$  of the peptide solution and  $45 \mu\text{L}$  of the DSPC solution in a vial. The solvent was first dried using a nitrogen flow, subsequently followed by exposure to vacuum for at least 16 hours. The dry material was hydrated by adding  $0.5 \text{ mL}$  PBS, heated to  $60^\circ\text{C}$  for 1 hour with intermittent vortexing, leading to liposomes with a final concentration of  $500 \mu\text{g}$  peptide per  $1 \text{ mL}$  of PBS. Finally, the suspensions were homogenized by sonication at  $60^\circ\text{C}$  for an hour.

## Spot-test to determine the presence of specific antibodies in serum.

Solutions were prepared of peptides **6**, **7** and **8** in milliQ water to a final concentration of  $0.5 \text{ mg mL}^{-1}$ . These solutions were diluted 2, 4, 8, 16 and 32 times. Nitrocellulose strips were cut from a sheet. On a strip a dilution series was applied by spotting with  $1 \mu\text{L}$  of each of the solutions in the series, the spot was allowed to dry in air. The strips were placed in a vessel which isolated each strip in a separate chamber. To each chamber  $2 \text{ mL}$  of blocking buffer (5% milk powder in a PBS buffer containing 0.2% Tween-20 (PBST)) was added, the vessel was agitated for 1 hour at RT. Subsequently the strips were washed 3 times for 5 minutes using PBST, in which they were kept moist until the next step.

To each vessel  $2 \text{ mL}$  of incubation buffer (2.5% milk powder in PBST) was added, and to the buffer  $5 \mu\text{L}$  of serum was added (500 times diluted). The strips were incubated overnight in the diluted serum at  $4^\circ\text{C}$ . The strips were subsequently washed 3 times for 5 minutes with PBST, in which they were kept moist until the next step. To the strips  $2 \text{ mL}$  solution of an anti-rabbit antibody labeled with horseradish peroxidase (HRP) 3000 times diluted in PBST milk (2.5 %) was added. Following 1 hour of incubation at room temperature the strips were washed again 3 times for 5 minutes using PBST. The washing buffer was removed and the strips were put on a glass plate. A freshly mixed solution of the HRP detection reagent mixture for the specific antibody was added to the strips and after 1 minute of incubation excess reagents were removed. The glass plate with the strips was covered with plastic foil and the whole set-up was quickly moved to a dark room. Photographic film was put on top of the glass plate and was developed after exposure. Films for the KLH sera were exposed for 1 minute, films for the liposomal sera were exposed for 5 minutes.

## SPR protocols

The Biacore 2000 was fitted with a carboxymethyl-dextran-derivatized CM5 research grade chip, following implemented protocols, priming the chip using HBS-EP running buffer (0.01 M HEPES [pH 7.4], 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20). Cyclic peptide **9** was dissolved in MilliQ to a concentration of  $4 \text{ mg mL}^{-1}$  and subsequently diluted to  $0.2 \text{ mg mL}^{-1}$  pH 5.0 using an acetate buffer. The peptide was immobilized on the chip using EDC (*N*-ethyl-*N'*-(dimethylaminopropyl)carbodiimide) and NHS (*N*-hydroxysuccinimide), both 1M aqueous solutions, in a standard coupling procedure.<sup>24</sup> Excess reactive groups were finally reacted with ethanolamine. A reference channel was coated with ethanolamine after EDC-NHS activation.

Binding of antibodies was determined at a HPB-EP buffer flow of  $20 \mu\text{L}/\text{min}$ . Serum was diluted with PBS and injected, and both peptide-functionalized and reference channels were monitored. The initial dilution factor of 50 was varied to obtain a binding curve.

Serums raised against **KLH-3** and **KLH-4** were diluted 12.5 times using PBS, serum raised against liposomes containing **2** was diluted 5 times in PBS. Solutions were prepared of peptides **6** and **9** in PBS to a concentration of 2, 0.2 and  $0.02 \text{ mM}$ . Serum dilutions and peptide solutions were mixed 1:1. These mixtures were injected and both peptide-functionalized and reference channels were monitored.

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## Summary

Proteins attain their specific properties by a three-dimensional folding and assembly process, which leads to the correct positioning of amino acids at for example recognition sites and active sites. In principle small peptides can be synthesized which mimic the amino acid sequence of a functional site of a protein, basically by cutting a piece out of the protein. However, these peptides lack the stabilizing effect of their spatial structure by the remainder of the protein, via *e.g.* hydrogen bonds, electrostatic interactions and hydrophobic interactions. In order to restore biological activity in these peptide fragments, numerous methods have been developed that ensure peptides to fold in the desired conformation, for example via the use of non-covalent interactions.

In this thesis it is examined whether peptide assembly and folding can be influenced by the introduction of additional hydrophobic interactions. The moieties used to increase the hydrophobicity were alkyl tails, which were introduced specifically at the peptide termini. Lipopeptides like these are a subset of peptide amphiphiles, and this set currently forms an important class of protein mimics and model systems as a result of their assembly behavior. In Chapter 1 an overview is presented on lipopeptide amphiphiles with a focus on their assembly behavior and resulting properties.

In Chapter 2 the influence of the introduction of alkyl tails to a self-assembling fibril forming peptide with the sequence Lys-Thr-Val-Ile-Ile-Glu is examined. The addition of a hydrophobic tail at the N-terminus of the peptide did not influence the morphology of the assemblies formed by the peptides in aqueous solution, as determined primarily using Circular Dichroism spectroscopy and Transmission Electron Microscopy. The stability of the assemblies was however increased by the introduction of the alkyl tail. Whereas fibrils formed by the acetylated peptide disassembled above 40°C and at a concentration below 60 µM, peptides with a tail of C12 or longer formed stable assemblies up to at least 90°C.

Since the introduction of hydrophobic interactions increased the stability of the peptide assemblies of Lys-Thr-Val-Ile-Ile-Glu, it was envisaged that increasing the solubility of the peptide in water should hinder the formation of fibrils. This was demonstrated in Chapter 3 through the synthesis of a peptide with a water-soluble polyethylene glycol (PEG) polymer at its C-terminus. Moreover, the introduction of a C16-tail to the N-terminus in conjunction with the C-terminal PEG resulted in a peptide that forms fibrils, since the



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additional hydrophobic interactions were able to overcome the solubilizing effect of the PEG chain. Interestingly, a minimum alkyl length was required to achieve stabilization, since tails up to C10 all resulted in non-assembling peptides, while the introduction of a C12 tail and longer resulted in the formation of fibrils that were stable up to at least 90 °C.

The presence of the two states, *i.e.* molecularly dissolved or in a fibrillar assembly, depending on hydrophobicity, opened the possibility to switch from assembled to dissolved peptides by specifically removing the hydrophobic tail. This was achieved by the introduction of a UV-cleavable linker between the pegylated peptide and the alkyl tail (C18). This molecule formed fibres in solution, which were, as expected, disassembled by exposure to UV light.

In Chapter 4, alkyl tails were introduced to the N-terminus of a peptide that does not self-assemble by itself but has a preferred fold in its native protein. This peptide, with the sequence Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly (GANPNAAG), is based on a sequence found in the malaria coat protein and is thought to be present in a  $\beta$ -turn conformation. The acetylated peptide did not have a preferred fold in solution, and the same was true for peptides with tails up to C12. In contrast, a tail of C14 or longer induced a  $\beta$ -sheet like conformation, which was concurrent with the appearance of fibrillar assemblies. These assemblies could be influenced by increasing the temperature, with a critical temperature of approximately 50 °C for the C14 and 80 °C for the C16 compound. The latter did retain some stabilization of the  $\beta$ -sheet-like conformation at these high temperatures, while the C18 compound still shows fibrils at this temperature. Also for these amphiphilic peptides a UV cleavable group was introduced between the stabilizing hydrophobic tail and the peptide, and the removal of the tail did result in the disassembly of fibrils.

Peptide amphiphiles can be co-assembled with phospholipids into liposomes. This was shown before for the C18-functionalized Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly peptide in DSPC liposomes (also containing C18 hydrophobic tails). In that case the peptide adopted a random coil structure. However, when the peptide was functionalized with C18 tails on both N- and C-terminus a  $\beta$ -turn conformation was obtained upon insertion in the DSPC liposome bilayer.

In Chapter 5 this methodology to stabilize the  $\beta$ -turn conformation of the peptide was further investigated, with the aim of tuning the stabilization. To this goal a series of peptides was synthesized with varying alkyl tail lengths, and to ensure anchoring, a second series was synthesized in which the C-terminal was always modified with a C18 tail and tails of varying length were coupled at the N-terminus. These peptides were first applied at the air-water

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interface of a Langmuir through. This showed that the presence of tails longer than C12 was needed to form a stable monolayer with the amphiphile in a  $\beta$ -turn conformation. Tails of C12 and shorter were forced into the aqueous subphase. The peptides were incorporated in liposomes composed of either DMPC, DPPC or DSPC (with C14, C16 and C18 tails respectively), in this case all peptides with at least one alkyl tail of C12 or longer were stabilized in a  $\beta$ -turn like structure. The stabilization was not influenced by the match between the peptide hydrophobic region and the liposomal bilayer.

As mentioned before the GANPNAAG sequence adopts a  $\beta$ -turn conformation in the native Malaria protein. Since GANPNAAG peptides incorporated in liposomes are forced to obtain a similar fold, it was of interest to investigate if this conformation resembled the native structure. This was tested in Chapter 6 by raising anti-bodies against the bifunctionalized C18 peptide in DSPC. The response against these liposomes was low, although the selectivity of the antibodies that were present was good. In order to increase the response a conjugate was made of the peptide functionalized with two hydrophobic tails and the carrier protein KLH. The hydrophobic tails were hoped to induce a stable  $\beta$ -turn to the peptide. Although the response was improved when compared to the liposomal system, the antibodies raised showed however low selectivity, probably as a result of a competing interaction between the hydrophobic tails and the carrier with the stabilization of the turn.



## **Samenvatting**

Eiwitten verkrijgen hun specifieke eigenschappen als gevolg van drie-dimensionale vouwing en complexatie, deze processen leiden tot de juiste positionering en oriëntatie van aminozuren bij bijvoorbeeld actieve sites en herkenning sites. In principe is het mogelijk om peptiden te maken die de aminozuurvolgorde van een functionele locatie kopiëren, vergelijkbaar met het knippen van dit stuk uit het eiwit. Maar bij deze peptiden ontbreekt de stabilisatie van een driedimensionale structuur door de rest van het eiwit, door o.a. waterstofbruggen, elektrostatische en hydrofobe interacties. Om de biologische activiteit van deze peptide fragmenten te behouden zijn er vele methodes ontwikkeld die er voor zorgen dat ze in de juiste conformatie vouwen, bijvoorbeeld door het gebruik van niet-covalente interacties.

In dit proefschrift wordt onderzocht of de vouwing en pakking van peptiden kan worden beïnvloed door de introductie van extra hydrofobe interacties. De functionele groepen die werden toegepast om het hydrofobe karakter te verhogen waren alkyl staarten, deze werden specifiek op het uiteinde van het peptide geïntroduceerd. Er zijn aanverwante peptide amfifielen bekend, en deze vormen als gevolg van hun aggregatie gedrag tegenwoordig een belangrijke klasse van eiwit-mimetica en modelsystemen. In Hoofdstuk 1 wordt een overzicht gegeven van lipopeptide amfifielen, voornamelijk gericht de structuren die ze vormen en de eigenschappen die daaruit volgen.

In Hoofdstuk 2 wordt de invloed van de introductie van een alkyl staart op de eigenschappen van een zelf-assemblerend, vezel vormend, peptide (Lys-Thr-Val-Ile-Ile-Glu) onderzocht. De morfologie van de structuren die door het peptide worden gevormd in een waterige oplossing werd niet beïnvloed door alkyl staarten die aan de N-terminus waren gekoppeld. Dit werd met name bepaald met behulp van Circulair Dichroïsme spectroscopie en elektronenmicroscopie. De stabiliteit van de gestructureerde aggregaten werd daarentegen wel beïnvloed door de introductie van de alkyl staart. Waar de structuren van het peptide zelf boven de 40 °C en bij concentraties lager dan 60 mM uit elkaar vielen bleven de structuren van het peptide met een C12 staart (of langer) heel tot minstens 90 °C.

Aangezien de introductie van extra hydrofobe interacties de stabiliteit van de structuren die werden gevormd door Lys-Thr-Val-Ile-Ile-Glu, leek het aannemelijk dat de introductie van een meer wateroplosbare groep de aggregatie zou remmen. Dit werd gedemonstreerd in Hoofdstuk 3 door een peptide te synthetiseren met een water-oplosbaar

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polyethyleenglycol (PEG) staart aan de C-terminus. Wanneer naast de C terminale PEG ook nog een C16 staart werd geïntroduceerd op de N-terminus vormde het resulterende peptide echter weer structuren, omdat de extra hydrofobe interacties sterk genoeg waren om de wateroplosbaarheid van de PEG te overwinnen. Interessant genoeg bleek een minimum lengte van de alkyl staart nodig om stabilisatie te bewerkstelligen, want staarten tot en met C10 leverden water-oplosbare peptiden op. Echter, zodra de lengte C12 of langer was werden structuren gevormd die zelf bij 90 °C nog stabiel waren.

Een klein verschil in een molecuul, een hydrofobe staart, kan dus een groot verschil in structuur opleveren, namelijk een oplosbaar molecuul of een gestructureerd aggregaat. Dit opent de mogelijkheid om van aggregaat naar opgelost molecuul te schakelen door de hydrofobe staart te verwijderen. Dit werd bewerkstelligd door tussen het PEG gefunctionaliseerde peptide en de alkyl staart (C18) een UV gevoelige schakel te introduceren. Het molecuul vormde gestructureerde aggregaten in oplossing, welke naar verwachting verdwenen onder invloed van UV licht.

In Hoofdstuk 4 wordt de introductie beschreven van alkylstaarten bij een peptide dat in water niet uit zichzelf aggregeert, maar in het eiwit waar het op gebaseerd is wel een voorkeursvouwing heeft. Dit peptide, met de sequentie Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly (GANPNAAG), is gebaseerd op een sequentie uit een oppervlakte-eiwit van malaria, waarin het waarschijnlijk een  $\beta$ -turn vouwing heeft. Het geacetylerde peptide zelf had in oplossing geen specifieke vouwing, en hetzelfde gold wanneer staarten met een lengte tot en met C12 werd geïntroduceerd. Voor langere staarten werd daarentegen een  $\beta$  sheet achtige vouwing waargenomen, en in dezelfde samples liet electronenmicroscopie fibers zien. Deze structuren konden worden beïnvloed door de temperatuur te verhogen. Bij een C14 staart was een temperatuur van 50 °C nodig en bij een C16 staart 80 °C, dit laatste molecuul hield zelfs bij deze hoge temperaturen echter nog een  $\beta$  sheet karakter. Tot slot behield het peptide met een C18 staart zelfs nog fibrillen bij 90 °C. Ook bij deze peptide amfifielen werd een UV gevoelige schakel ingebouwd tussen peptide en alkyl staart. Dit peptide vormde structuren, welke onder UV belichting verdwenen.

Peptide amfifielen kunnen samen met fosfolipiden co-assembleren in liposomen. Dit was aangetoond voor het Gly-Ala-Asn-Pro-Asn-Ala-Ala-Gly peptide gefunctionaliseerd met een C18 staart, en DSPC (C18 staarten). In dit geval liet het ingebouwde peptide een willekeurige vouwing zien. Daarentegen werd, als het peptide aan beide termini met een C18

staart werd gefunctionaliseerd, het peptide na incorporatie in de bilaag van een DSPC liposoom gestabiliseerd in een  $\beta$  turn.

Deze methode om een  $\beta$  turn te stabiliseren werd in Hoofdstuk 5 verder uitgewerkt, met als doel de stabilisatie beter te kunnen sturen. Hiertoe werden peptiden gesynthetiseerd met in lengte variërende alkyl staarten. Verder werd, om zeker te zijn van ankering in het liposoom, een serie peptiden gemaakt waarbij de C-terminus altijd met een C18 staart was gefunctionaliseerd terwijl staarten met een variatie aan lengtes werd gekoppeld aan de N-terminus werd gevarieerd. Deze peptiden werden aangebracht op de water-lucht grens in een Langmuir-trog. Hieruit bleek dat beide staarten langer dan C12 moesten zijn om een stabiele monolaag te vormen waarin het peptide in een  $\beta$  turn conformatie was gevouwen. Staarten met een lengte van C12 of korter werden tijdens compressie in de waterfase gedwongen. De peptiden werden ingebouwd in liposomen van DMPC, DPPC dan wel DSPC (met C14, C16 of C18 staarten), en hierbij werden alle peptiden met minstens een staart van C12 of langer gestabiliseerd in een  $\beta$  turn achtige vouwing. De stabilisatie werd niet beïnvloed door (gebrek aan) overeenkomst tussen de hydrofobe staart van het peptide en de lipide bilaag.

Zoals hierboven staat beschreven is de GANPNAAG sequentie in het natuurlijke eiwit in een  $\beta$  turn conformatie aanwezig. Omdat de GANPNAAG peptiden in een liposoom in deze zelfde conformatie worden gedwongen was het interessant om te bepalen of deze conformatie de natuurlijke vouwing benaderde. Dit werd in Hoofdstuk 6 getest door antilichamen op te wekken tegen het liposomen van DSPC waarin het peptide met twee C18 staarten was ingebouwd. De immuunrespons tegen dit systeem was laag, maar de opgewekte antilichamen waren wel specifiek. Om de respons te verhogen werd een peptide met twee hydrofobe staarten geconjugeerd aan KLH, een drager eiwit, waarbij de hydrofobe staarten de  $\beta$  turn zouden moeten stabiliseren. Hoewel de respons hoger was dan bij het liposomale systeem werd er een lagere specificiteit waargenomen. Vermoedelijk was dit een gevolg van competitieve interactie tussen de hydrofobe staarten met het drager eiwit.



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## **Dankwoord**

Na de jaren van een promotieonderzoek en in mijn geval van schrijven rest nog een belangrijk deel van ieder proefschrift. Want gedurende al die jaren waren er mensen die steunen, motiveren, afleiden, helpen... en zonder hen was het voltooien van dit proefschrift onmogelijk geweest. Natuurlijk wil je daarvoor iedereen bedanken, en bij deze wil dat ook zeker doen, maar het is niet altijd makkelijk om goed onder woorden te brengen, en om het goed te doen zou het aantal pagina's in dit proefschrift makkelijk kunnen verdubbelen. Toch zal ik hier een poging wagen, hoewel ik natuurlijk hier en daar dingen en zelfs mensen zal vergeten te noemen, sorry maar in ieder geval heel erg bedankt.

In eerste instantie ben ik natuurlijk mijn promotor Jan van Hest dankbaar voor de begeleiding tijdens mijn onderzoek en zeker tijdens het schrijven dat er ook bij hoort. Je hebt me toch iedere keer weer weten te motiveren en interesseren, en ik ben nog steeds onder de indruk van de manier waarop je de juiste woorden weet te vinden om orde te brengen in een relatieve chaos aan resultaten en ideeën. Ook wil ik Bert Meijer bedanken, vanzelfsprekend voor de samenwerking die mijn promotie mogelijk maakte, ik blijf het jammer vinden dat ik daar niet meer mee heb kunnen doen. Maar ook voor de tijd die ik tijdens mijn afstuderen in je groep heb doorgebracht.

In de dagelijkse begeleiding was daar Dennis Löwik, een bron van inspiratie en ideeën en aangenaam snel als het op nakijken aankomt. Ik kan ondertussen binnen 20 minuten een reactie inzetten, maar helaas moet ik nog wel de truc leren om daar niet eerst dagen over na te denken. In het algemeen geldt dat ik gezegend ben geweest met begeleiders en collega's waarbij de deur altijd openstaat en dat geldt zeker voor jou.

Toen ik begon aan deze promotie wist ik nauwelijks iets van peptide-chemie, gelukkig was daar Hans Adams. Niet alleen vond ik in het begin een plek in je kantoor, maar je hebt me ook de ontspannen houding bijgebracht die in ieder geval synthese van peptiden tot een goed einde brengt. Ik vermoed dat ik maar een fractie van je kennis en trucs heb kunnen overnemen, maar dat is al voldoende om een heel eind te komen. Een brainstormsessie met jou en Dennis was vaak voldoende voor 5 strategieën die horen te werken en 10 routes die in ieder geval hopeloos zijn. Ik zal in ieder geval in mijn achterhoofd houden dat heel veel dingen eigenlijk al jaren geleden zijn uitgezocht en dat je in ieder geval dingen gewoon moet uitproberen.



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Iemand die hier niet mag ontbreken is Marjolijn Roeters, die de basis heeft gelegd onder een belangrijk deel van dit proefschrift. De reden dat dit heeft kunnen gebeuren is dat je de dappere keuze hebt gemaakt om je eigen promotie te staken om je geluk elders te vinden. Jouw werk, en dat van je studenten, vormt de kern van de resultaten in hoofdstuk 2 en daarmee de inspiratie voor het werk beschreven in hoofdstuk 3.

Het werk beschreven in hoofdstuk 6 had niet kunnen plaatsvinden zonder de hulp van prof. Stunnenberg en van zijn afdeling Moleculaire Biologie. Met name de hulp van Josephine Jansen, Anita Kaan en Adriana Salcedo was groot en zo nu en dan onmisbaar. In hetzelfde project was de samenwerking met Hinke Malda van de TU Eindhoven belangrijk, haar antilichamen tegen vergelijkbare peptiden werkten zo nu en dan beter dan die van mij en hebben een flink aantal tests mogelijk gemaakt die dit boek helaas niet hebben gehaald.

Het werk in het lab kan niet zonder de nodige ondersteuning. Binnen de afdeling is er natuurlijk het secretariaat met Jacky (bedankt voor de motivatie, die nu en dan nodig was) en ook Désirée en Paula. Voor apparatuur, bestellingen en onderhoud waren Peter, Pieter, Peter, Ad en Helene altijd bereikbaar. Hoewel we veel hebben is toch niet alles aanwezig, dus wil ik ook Huub op den Camp (Maldi-TOF), Liesbeth, Rien, Geert-Jan (algemeen instrumentarium) Will Roefen en Meta Roestenberg bedanken voor de hulp bij verscheidene metingen.

Natuurlijk alle collega's die de afgelopen jaren zowel tijdens mijn promotie als daarna het deel uitmaken van de groep van Jan zo'n plezier maken. Met name Dennis, Jurgen, Lee, Henri, Joost, Marjolijn, Eric, Mark, Pieter, Kaspar, Rosalie, Linda, Hefziba, Stijn, Luiz, Sanne, Maddi, TuHa, Maaïke, Ruud, Dennis, Morten, René, K.T., Sander en bovendien alle studenten.

Tijdens mijn promotie heb ik een aantal studenten mogen begeleiden, waarbij niet iedereen technisch gesproken voor mij werkte, maar dat wil niet zeggen dat ik er niet van heb genoten. Bovendien heb er veel van geleerd, wat wel als nadeel moet hebben gehad dat mijn begeleiding niet altijd even helder moet zijn geweest daarvoor (nogmaals) mijn verontschuldigheden. Jaime en Loes hebben sterk bijgedragen aan het ontdekken van de eigenschappen van de GANPNAAG vezels. Remon en Inge hebben in aparte projecten de basis gelegd voor het gebruik alhier van de UV-kliefbare linkers voor het schakelen van peptide eigenschappen en structuren, waarbij moet worden aangemerkt dat hun eigen resultaten hoewel ze niet in dit proefschrift voorkomen nu de basis vormen voor het onderzoek van een promovendus. Een groot deel van de begeleiding van jullie stages lag dan wel bij Dennis, maar ik ben toch erg dankbaar voor jullie bijdragen.

Evelyn heeft veel werk verricht aan de validering van de ligatie van lipopeptiden aan KLH, het blijft toch eng om te zien hoe kort dat kan worden opgeschreven. Maar de gemoedsrust die je resultaten gaven is niet in woorden te omvatten. Valentina, you suddenly (to me) appeared fom Italy, and your efforts were an important contribution to the publication of the results in Chapter 2. I enjoyed your presence and suspect you had a nice time here. Marloes, je hebt in ieder geval hier geleerd dat organische chemie je ding niet is, en ik hoop nog steeds dat die conclusie niet aan mij ligt. Maar je inspanningen en resultaten hebben toch bijgedragen aan Hoofdstuk 3, dus je kunt het in ieder geval wel. Iris en Wouter hebben kortere stages doorgebracht en hebben wat zijwegen getest die helaas nergens op zijn uitgelopen. Naast de reguliere studenten waren er ook nog de MLW practica, het blijft verbijsterend hoe efficiënt je dingen kunt uitzoeken als alles strak is gepland, Peter, Inez, Lisa, Kwinten hebben met Henri zelfs een gepubliceerd deel bijgedragen aan hoofdstuk 4.

In in de loop der jaren ben ik in contact gekomen met velen binnen de (an)organische afdelingen. In eerste instantie begonnen op het peptiden-lab op de 1<sup>e</sup> verdieping, met alle interessante contacten met de personen in de andere labs die daar bij zaten en natuurlijk de hele Nolte-groep waarmee koffie werd gedronken. Koffie boven drinken en samen lunchen was belangrijk in het contact met de rest van de groep van Jan, en ook met de groep van Floris en de mensen van Chiralix. De verhuizing van het UL naar het Huygensgebouw veranderde wat interacties en versterkte velen. Zoals mensen misschien is opgevallen ben ik ben redelijk nieuwsgierig, ook in het lab, waardoor ik bij vele mensen over de schouder heb meegekeken, en hen vaak ook heb proberen te helpen. Zelf heb ik en veel plezier aan beleefd en en een en ander geleerd. Ik hoop dat een deel van mijn bemoeienis uiteindelijk nuttig is geweest.

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Familie is er altijd, en ik ben iedereen erg dankbaar voor alle steun. Ik hoop dat ik nu duidelijk kan uitleggen wat ik nu eigenlijk de afgelopen jaren heb gedaan, hoewel jullie de juiste termen ondertussen al lijken te kennen. Mieke, bedankt voor alles, de afgelopen jaren altijd klaarstaand, zeker voor de morele ondersteuning. Han, bedankt, je laat me mijn eigen weg volgen maar hebt hem zeker ook mogelijk gemaakt. Janne, Femke en Gijs en Olga, bedankt voor afleiding, er gewoon zijn en alle gesprekken en leuke dingen die we hebben gedaan.

*Joris*

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## ***Curriculum Vitae***

Joris Meijer werd geboren op 26 Mei 1976 te Enschede. Na het behalen van zijn VWO diploma aan het Van Maerlantlyceum te Eindhoven startte hij in 1994 met een studie scheikunde aan de Universiteit Utrecht, welke in 2002 werd afgerond met een diploma. Tijdens de studie werd het afstudeeronderzoek ‘supramolecular binding to surfaces’ uitgevoerd in de groep van prof. dr. Bert Meijer aan de Technische Universiteit Eindhoven. Hierna begon Joris aan een promotieonderzoek, onder leiding van prof. dr. ir. Jan van Hest en dr. Dennis Löwik, in de groep bio-organische chemie aan de Radboud Universiteit Nijmegen (indertijd Katholieke Universiteit Nijmegen). De belangrijkste resultaten van dit onderzoek zijn in dit proefschrift beschreven. Momenteel is Joris werkzaam als post-doctoraal onderzoeker in de groep van prof. van Hest.